

From the department of Physiology & Pharmacology
Karolinska Institutet, Stockholm, Sweden

Exercise and regulation of metabolic function in human skeletal muscle

with special reference to PGC-1 α and
the mitochondria

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**Karolinska
Institutet**

STOCKHOLM 2017

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Published by Karolinska Institutet.

Printed by Eprint AB 2017

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ISBN XXX-XX-XXXX-XXX-X

Exercise and regulation of metabolic function in human skeletal muscle

with special reference to PGC-1 α and
the mitochondria

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

*“Anyone who is not confused about oxidative phosphorylation
just doesn’t understand the situation”*

Efraim Racker

ABSTRACT

Regular physical activity is highly associated with many health benefits and regular exercise training is used in the prevention and treatment of a large number of disease conditions, including type 2 diabetes, cardiovascular disease and cancer. One of the key adaptations to regular exercise training is mitochondrial biogenesis and improved oxidative capacity, particularly in skeletal muscle tissue. There is an inverse relationship between the dose of regular physical activity and the risk for premature death and this might in part be explained by the mitochondrially related improvement of metabolic health in trained skeletal muscle. In contrast, low whole body aerobic capacity and muscle mitochondrial content are characteristics of a sedentary lifestyle that contribute to the development of metabolic disease and other disorders. Most tissues adapt to exercise training, not least skeletal muscle, which is a highly plastic tissue. Cellular adaptations in skeletal muscle are driven by extra- and intracellular signals arising from the exercise stimulus, *e.g.* changes in shear stress, oxygen tension, energy levels, pH and temperature. Ultimately, these cellular perturbations lead to gene expression and protein alterations that improve skeletal muscle, *e.g.* through enhanced mitochondrial function.

The results in this thesis are based on skeletal muscle biopsies from the *m.vastus lateralis*, taken at rest (all studies) and at 30 min (study 2 and 3), 2hrs , 6hrs and 24 hrs (study 3) after an acute bout of exercise or after three months of training (study 4). The study subjects in Studies 1-3 were young healthy normally active individuals while in study 4 older men with impaired glucose regulation was recruited. Four different experimental models were used in this thesis; first, a one-legged knee extension exercise model with or without restricted blood flow in the leg; second, an acute bout of 60 min cycling; third, an acute bout of 60 min cycling (humans) or 36-40 min running on a treadmill (mice) or a 12-weeks high fat diet intervention (mice); and last a 12-week intervention in which resistance training or Nordic walking was performed.

The main focus of this thesis was on the transcriptional coactivator PGC-1 α and its upstream and downstream targets, coactivators and corepressors and how all these are affected by exercise. In Paper I, we show for the first time that PGC-1 α can be transcribed from an alternative promotor in human skeletal muscle and that the PGC-1 α -ex1b transcript seems like the most avidly exercise-induced transcript. In brief, an acute exercise bout with restricted blood flow massively increased the mRNA levels of the human skeletal muscle PGC-1 α splice variant 2 hours after exercise, most likely mediated through activation of an

alternative promoter. Protein data supported previous studies demonstrating the importance of AMPK activation in exercise-induced expression of PGC-1 α mRNA. In paper III, twenty-two mRNA transcripts and five proteins were measured over a 24 h time-course. Interestingly, as a response to exercise the protein levels of PGC-1 α -ex1b increased before the elevation of the Total PGC-1 α protein which might indicate its importance in the early adaptation processes. We also demonstrated for the first time the existence and post-exercise expression pattern of two LIPIN-1 (LIPIN-1 α and LIPIN-1 β) and three NCoR1 (NCoR1-1, NCoR1-2, and NCoR1-3) isoforms in human skeletal muscle. And just as in Paper I the data emphasized PGC-1 α -ex1b as the most exercise-responsive PGC-1 α isoform. In Paper II, the investigation aimed to define a functional role for BRCA1 in skeletal muscle using a translational approach. For the first time, BRCA1 and two shorter isoforms were identified in both humans and mouse skeletal muscle. In response to exercise, an increased interaction between BRCA1 and ACC-p was seen in both humans and mice. Decreasing the content of BRCA1 in primary human myotubes resulted in decreased oxygen consumption by the mitochondria and increased reactive oxygen species production. The decreased BRCA1 content also resulted in increased storage of intracellular lipids and reduced insulin signaling in human myotubes. These results indicate that BRCA1 might play a critical role in the regulation of metabolic function in skeletal muscle and address BRCA1 as a novel target to study further to pursue metabolic diseases. Lastly, Paper IV shows for the first time that protein levels of the mitochondrially encoded and derived peptide humanin increases after 12-weeks of regular resistance exercise. This very small peptide has been implied to have multiple functions including neuroprotective effect and a positive effect on glucose metabolism and oxidative stress. Preliminary data from the same study material also revealed that MOTS-c, another small mitochondrially derived and encoded peptide, from the 12S rRNA gene, also seems to be affected by resistance training. These mitochondrially encoded peptides are interesting target to study further in the attempt to understand, and in the future to optimize, retrograde signaling and maybe use them to treat diseases in which mitochondrial function is impaired, e.g. type 2 diabetes, Alzheimer's disease and cancer.

In conclusion, regular endurance training increases mitochondrial density through a complex network of transcriptional regulators that in an accumulated way are affected by each single exercise bout, and therefore is acute exercise also important to study in the endeavor to comprehend mitochondrial adaptations. Thus, it is important from a clinical, as well as basic science perspective to understand the regulation of skeletal muscle gene activity and the adaptation process at a molecular level in an attempt to recognize how it might contribute to the many health benefits seen with a physically active lifestyle.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

- I. J. Norrbom, **E-K. Sällstedt**, H. Fischer, C. J. Sundberg, H. Rundqvist and T. Gustafsson. *Alternative splice variant PGC-1 α -b is strongly induced by exercise in human skeletal muscle*. Am J Physiol Endocrinol Metab, 2011, 301: E1092-E1098.
- II. K. C. Jackson, **E-K Gidlund**, J. Norrbom, A. P. Valencia, D. M. Thomson, R. A. Schuh, D. P. Neuffer, E. E. Spangenburg. *BRCAL is a Novel Regulator of Metabolic Function in Skeletal Muscle*. J Lipid Res, 2014, 55(4), p.668-680.
- III. **E-K Gidlund**, M. Ydfors, S. Appel, H. Rundqvist, C. J. Sundberg and J. Norrbom. *Rapidly elevated levels of PGC-1 α -b protein in human skeletal muscle after exercise: exploring regulatory factors in a randomized controlled trial*. J Appl Physiol, 2015 119: 374-384.
- IV. **E-K Gidlund**, F. von Walden, M. Venojärvi, U. Risérus, O. J. Heinonen, J. Norrbom and C. J. Sundberg. *Humanin skeletal muscle protein levels increase after resistance training in men with impaired glucose metabolism*. J Phys Rep, 2016, 4(23),e13063.p 1-10

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LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ACC-p	Phosphorylated form of acetyl CoA carboxylase
Acetyl-CoA	Acetyl Coenzyme A
ADP	Adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide ribofuranoside
Akt/PKB	Akt/protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-dependent protein kinase
ANOVA	Analysis of variance
AS160	Akt substrate of 160 kDa, a.k.a TBC1D4
ATP	Adenosine triphosphate
BRCA1	Breast cancer 1 early onset
Ca ²⁺	Calcium ions
CaMK	Ca ²⁺ -calmodulin-dependent protein kinase
CAT	Carnitine translocase
CO	Carbon monoxide
CO ₂	Carbon dioxide
CPT1 / CPT2	Carnitine palmitoyltransferase 1/ 2
CREB1	cAMP responsive element-binding protein-1
CS	Citrate synthase
D-loop	Displacement loop
DNA	Deoxyribonucleic acid
EIM	Exercise is medicine
ERR	Estrogen-related receptors
ETC	Electron transport chain

FABP	Fatty acid binding protein
FACS	Fatty acyl-CoA synthase
FADH ₂	Flavin adenine dinucleotide
FAT/CD36	Fatty acid translocase
FATP	Fatty acid transport protein
Fe ²⁺	Iron ions
GLUT4	Glucose transporter type 4
H ⁺	Hydrogen ions
HATs	Histone acetyltransferases
HbA1c	Glycated hemoglobin
HDACs	Histone deacetylases
HDL	High-density lipoprotein
HFD	High fat diet
HIF-1	Hypoxia inducible factor -1
HIIT	High Intensity Interval Training
HN	Humanin
HS1 / HS2	Heavy strand 1 / heavy strand 2
IGF	Impaired fasting glucose
IGFBP- 3	Insulin-like growth factor binding protein 3
IGR	Impaired glucose regulation
IGT	Impaired glucose tolerance
IRS-1	Insulin receptor substrate 1
LIPIN-1	Lipid metabolism enzyme a.k.a LIPIN1
MaCoA	Malonyl-CoA
MAPK	Mitogen-activated protein kinase
MCD	Malonyl CoA decarboxylase
MCIP1	Myocyte-enriched calcineurin-interacting protein-1

MDP's	Mitochondria-derived peptides
MEF2	Myocyte enhancer factor-2
MHC	Myosin heavy chain
MOTS-c	Mitochondrial open reading frame of the 12S rRNA-c
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
MTERFs	Mitochondrial transcription termination factors
mtSSB	Mitochondrial single-stranded DNA-binding protein
NADH	Nicotinamide adenine dinucleotide
NCD	Non-communicable disease
NO	Nitric oxide
NCoR1	Nuclear Receptor Corepressor 1
NRF-1 / NRF-2	Nuclear respiratory factors 1 / 2
NUMTs	Nuclear genome insertions of mitochondrial origin
OCR	Oxygen consumption rates
OGTT	Oral glucose tolerance test
OH	Origin of heavy-strand replication, a.k.a OriH
OL	Origin of light strand replication site
p53	Tumor protein p53
PDK	Phosphoinositide-dependent kinase
PGC-1 α	Peroxisome proliferator-activated receptor γ co-activator-1 α
PI3-K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
p53	Tumor protein p53
PDK	Phosphoinositide-dependent kinase
PGC-1 α	Peroxisome proliferator-activated receptor γ co-activator-1 α
PI3-K	Phosphatidylinositol 3-kinase

PKC	Protein kinase C
POLG	Polymerase γ
POLRMT	Mitochondrial RNA polymerase
PPAR	Peroxisome proliferator-activated receptor
Rac1	Ras-related C3 botulinum toxin substrate 1
RNA	Ribonucleic acid
RIP140	Nuclear Receptor Interacting Protein 1, a.k.a NRIP1
ROS	Reactive oxygen species
RQ	Respiratory quotient
rRNA	Ribosomal RNA
SDH	Succinate dehydrogenase
SDHA	SDH subunit-A
T2DM	Type 2 diabetes mellitus
TBC1D1	TBC1 domain family member 1
TCA	Citrate acid cycle or tricarboxylic acid cycle
TFAM	Mitochondrial transcription factor A
TFB1M / TFB2M	Mitochondrial transcription factor B1/ B2
TIM	Translocase of the inner membrane
TOM	Translocase of the outer membrane
tRNA	Transfer RNA
UKK	Urho Kaleva Kekkonen Institute (the UKK Institute) 2-km walking test
VEGF	Vascular endothelial growth factors
VO ₂ max	Maximal oxygen consumption
VO ₂ peak	Peak maximal oxygen consumption

THESIS AT A GLANCE

Paper	Aim	Results	Conclusion
I	To explore acute exercise- induced expression of PGC-1 α and its different transcripts.	PGC-1 α -ex1a and PGC-1 α -ex1b increase in gene expression with exercise . AICAR and Norepinephrine increased expression of PGC-1 α -ex1a and PGC-1 α -ex1b.	PGC-1 α -ex1a and PGC-1 α -ex1b transcribed from alternative promoters are present and change with acute exercise. AMPK seem like a potential activator and PGC-1 α -ex1b like the most exercise-responsive transcript.
	To examine and explore the presence and function of BRCA1 in skeletal muscle and its response to acute exercise.	BRCA1 total and the two shorter isoforms, BRCA1 Δ 11, BRCA1 Δ 11b, are present in both human and mice skeletal muscle. Exercise increases the interaction between BRCA1 and ACC-p. Silencing BRCA1 impaired fat oxidation, glucose uptake and mitochondrial function.	BRCA1 is an important regulator of skeletal muscle metabolism and exercise seems to stimulate its action.
III	To investigate if PGC-1 α and its different transcripts and regulatory factors are regulated over a 24 hour time course.	PGC-1 α -ex1b gene and protein expression are highly and rapidly affected by exercise.	The results from the study indicate that PGC-1 α -ex1b is the most exercise-responsive PGC-1 α isoform.
VI	To study the mitochondrially encoded peptide humanin after 12 weeks of training in men with impaired glucose regulation.	Humanin increases with resistance training in skeletal muscle homogenate but not in serum.	Humanin levels increase with regular resistance training in prediabetic males and seems to have a role in glucose metabolism.

1 BACKGROUND

1.1 PHYSICAL ACTIVITY AND HEALTH

1.1.1 The benefits of exercise

Movement and physical activity has always been a natural part of human life and physiology. Historically, the ability to perform physical activity was essential for survival. The connection between regular physical activity, endurance capacity and health was documented as early as 3 000-2 500 BC in China and India (MacAuley 1994; Tipton 2014).

Since the middle of the 20th century, a growing body of research has investigated the influence of regular physical activity on some of the most common diseases such as type 2 diabetes, cardiovascular disease and Alzheimer's disease (Morris et al. 1953; Warburton et al. 2006; Booth & Roberts 2008; Booth et al. 2012). Even though we now know much about the very strong positive effects of exercise, physical inactivity, obesity and metabolic diseases are becoming much more common. They contribute to a severe and increasing burden of so-called non-communicable disease (NCD) globally impacting individuals and society. Regular physical activity has many well documented salutary effects on health and performance, such as improved cardiovascular function, improved oxidative capacity, increased insulin sensitivity and improved quality of life (Holloszy & Booth 1976; Booth & Roberts 2008; Chimen et al. 2011; Russell et al. 2014). There is a strong inverse relation between total dosage (frequency, intensity and duration) of regular physical activity and risk for overall morbidity (Byberg et al. 2009; Blair 2009; Barry et al. 2014; Bouchard et al. 2015). Also, endurance training positively influences for example the endocrine system (Henriksson 1995), the nervous system (van Praag 2009; Gallaway et al. 2017), and even promotes a better mental health (Fox 1999) as well as attenuates aging of the skin (Crane et al. 2015). In summary, physical activity influences the whole body, from the inside to the outside in beneficial ways.

Advancing technologies such as the use of transgenic models, whole genome sequencing, transcriptomics and bioinformatics have been introduced. They have led to the identification of genes and their corresponding proteins which are affected by physical activity and also genes involved in different diseases (Baralle & Buratti 2017; Kienzler et al. 2017). Studies have shown that obesity and type 2 diabetes are associated to a dysfunction of skeletal muscle mitochondria (Mazat et al. 2001; Kelley et al. 2002; Patti et al. 2003; Chansemaume et al. 2006; Chanséaume et al. 2007). Also, genes involved in mitochondrial oxidative

phosphorylation are less expressed in muscle of insulin-resistant patients suffering from obesity or type 2 diabetes (Simoneau & Kelley 1997; Kelley et al. 2002; Patti et al. 2003; Petersen et al. 2004; Ritov et al. 2005; Petersen et al. 2005). Despite that hundreds of papers have been published on mitochondrial biogenesis, it may represent only the tip of the iceberg regarding the biology of the mitochondrion, its oxidative capacity and the relationship to metabolic regulation.

1.1.2 Metabolic dysregulation

Our modern societies are facing a large global increase in metabolic disorders, linked largely to inactivity, inappropriate nutritional and other lifestyle habits as well as genetic predisposition. Metabolic dysregulation is by definition an impairment of a physiological regulatory mechanism. In this thesis, men with impaired glucose regulation (IGR) were studied before and after a training intervention. People with IGR, as defined by the American diabetes association, are individuals having impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG) which can collectively be termed prediabetes (Anon 1997; American Diabetes Association 2010).

Factors such as inactivity and obesity are often the initial triggers for the dysregulation and metabolic disorders, usually characterized by high levels of triglycerides and low levels of serum high-density lipoprotein (HDL) (Desvergne et al. 2006). Glucose levels in the blood in the postprandial period or upon fasting are normal at an early stage of metabolic dysregulation, reflecting a good β -cell function. If the dysregulation progresses, IGT, are usually diagnosed. This is characterized by an increased postprandial hyperglycemia and insulinemia (Desvergne et al. 2006). Type 2 diabetes mellitus (T2DM) occur as one of the later stages in this disease progression. All these different stages of metabolic dysfunctions are responsive to lifestyle changes and/or pharmacological treatment aiming to control glucose levels, insulin resistance, dyslipidemia, and the vascular complications.

As mentioned in section 1.1, there are tremendous benefits of being regularly physically active, not least when it comes to prevent and treat a metabolic dysregulation and T2DM (Eriksson & Lindgärde 1998; Tabák et al. 2012; Bansal 2015; Koh 2016; Hesselink et al. 2016). Exercise training improves glucose tolerance and insulin action in persons with IGR, as well as in T2DM (Rogers et al. 1988; Hughes et al. 1993). Substantial evidence suggest that the molecular mechanism for enhanced glucose uptake in the skeletal muscle with

exercise training may be due to increased expression and activity of key proteins coupled to metabolic signaling (Zierath 2002; Desvergne et al. 2006; Colberg et al. 2010). It is well established that dysfunctional glucose handling seldom comes without impairment in fat oxidation, and therefore it is important to study both of these metabolic pathways when evaluating prediabetes patients (Randle et al. 1963).

1.2 SKELETAL MUSCLE

Skeletal muscle tissue constitutes between 40 and 50 % of total body mass, making it the largest organ in the body. Skeletal muscle can be seen as a multifaceted organ. Besides enabling locomotion, it is also a highly metabolically active tissue involved in the regulation of nutrients such as glucose and electrolytes *e.g.* potassium and calcium, and also serves as a protein reservoir (Fitts et al. 1975; Holloszy & Booth 1976). Skeletal muscle is the primary target for insulin-dependent and non-dependent glucose uptake, and accounts for up to 80 % of glucose disposal under insulin-stimulated conditions, with marked impact on glucose homeostasis (DeFronzo et al. 1981; E. A. Richter & Hargreaves 2013). Additionally, skeletal muscle is the largest glycogen storage organ, with almost a 4-fold higher capacity than that of the liver (Mikines et al. 1988; Koopman et al. 2006).

Within this multifaceted capacity, skeletal muscle is also suggested to act as a regulatory endocrine organ, contributing with its own secretome (Weigert et al. 2014; Karstoft & B. K. Pedersen 2016). The secretome of the muscle consists of several hundred peptides (called myokines) that may be involved in communication between skeletal muscle and other tissues such as adipose tissue, liver, pancreas, bone, and the brain (L. Pedersen & Hojman 2012; Benatti & B. K. Pedersen 2015; Karstoft & B. K. Pedersen 2016). It is possible that myokines in part mediate the preventative effects of exercise against chronic diseases, such as T2DM, cardiovascular diseases, cancer, and dementia. Interestingly, myokines have been implicated in mediating the paradigm of exercise as medicine (B. K. Pedersen & Saltin 2015). By combining experimental and bioinformatics approaches it was recently shown that insulin-resistant skeletal muscle cells contained over 1000 putative secretable proteins, including some growth factors and cytokines. Important from a clinical perspective is that approximately 40 % of the secretable proteins found in these skeletal muscle cells were regulated under insulin-resistant conditions (Deshmukh et al. 2015).

It is commonly accepted that metabolic regulation in the skeletal muscle of complex organisms usually relies on different types of control systems. This control systems includes binding of an activator to a target, posttranslational modifications, protein-protein interactions and transcriptional regulation (Desvergne et al. 2006). This thesis touches upon some of these regulatory mechanisms in skeletal muscle in response to physical activity and in metabolic dysfunctional stages.

1.2.1 Skeletal muscle structure and plasticity

Human skeletal muscle fibers are long, large and multinucleated cells that are highly organized into fiber bundles. Skeletal muscle fibers (cells) differ considerably in their metabolic profile (oxidative and glycolytic enzyme capacity), contractile speed and fatiguability, reflecting their ability to adapt to and cope to different demands and lifestyles.

The dominating system for classifying human skeletal muscle fibers is based on isoforms of the myosin heavy chain (MHC) (Essén et al. 1975; Spangenburg & Booth 2003). In humans, there are three different types of skeletal muscle fibers namely type I, type IIa and type IIx. The type I fibers are slow-twitch oxidative fibers that can produce force over a long time/duration. Both type IIa and IIx are fast-twitch, glycolytic fibers that have high specific force production but low endurance capacity (Bárány 1967; Essén et al. 1975; Spangenburg & Booth 2003). With increasing contractile force, the recruitment of type IIa is prioritized before the type IIx (Egan & Zierath 2013). Skeletal muscle can be seen as a Russian doll, with layers and layers of smaller units that make up the full functional unit (see Fig. 1).

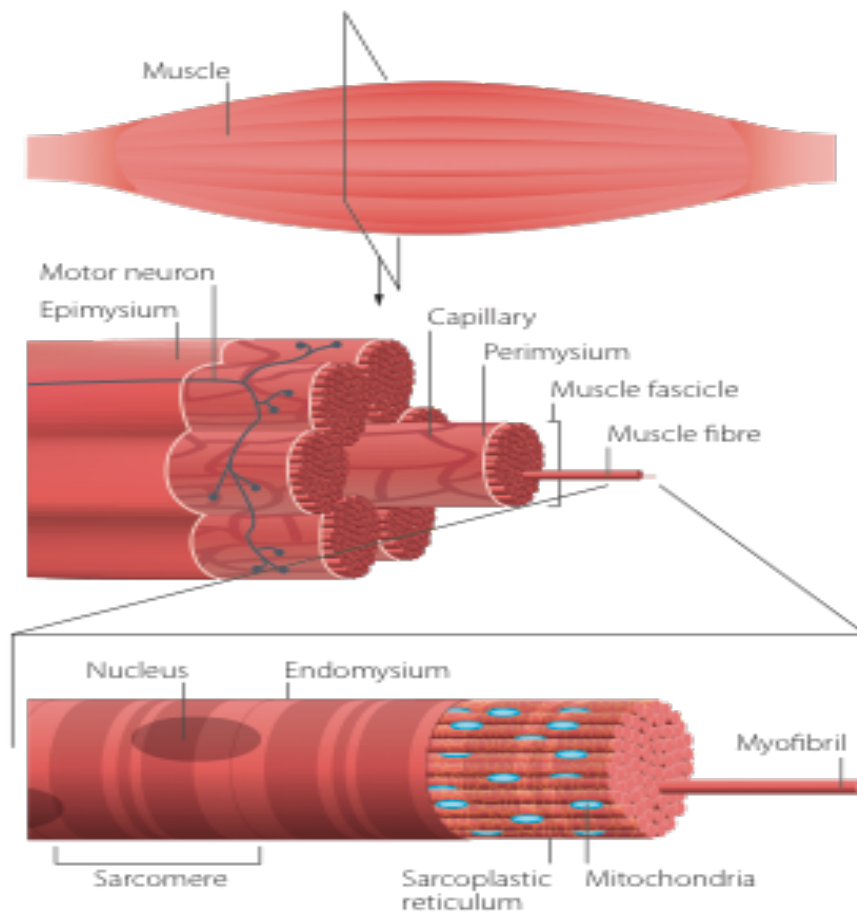


Figure 1. Schematic illustration of human skeletal muscle structure. Each muscle contains several bundles of individual skeletal muscle cells (fibers). The individual muscle fibers are surrounded by capillaries (dark red). An organized pattern of contractile myofibrils can be found inside the fibers, along with ATP-producing mitochondria (light blue), several nuclei and the Ca^{2+} -containing sarcoplasmic reticulum (yellow). *Illustration: Mattias Karlén*

Each skeletal muscle fiber contains multiple myofibrils, in which the sarcomere, the smallest and highly organized contractile unit is located. The fibers are arranged in parallel to each other and organized in bundles surrounded by connective tissue (the fascia). Each sarcomere contains the two main myofilaments, the proteins actin and myosin. By sliding against each other, and by the action of the cross-bridges, the myofilaments ultimately produce the mechanical force that contracts the muscle. The myofiber is surrounded by the sarcoplasmic reticulum that stores Ca^{2+} for release upon activation of the muscle. Ca^{2+} is the key that unlocks the contractile proteins and thus initiates the contractile process. In brief, Ca^{2+} makes the muscle contract and then relax in coordination with the provision of adenosine triphosphate (ATP). Going from rest to full activity can induce a 20-fold increase in whole-body metabolic rate (Hale 2008; Egan & Zierath 2013) and increase energy demand >100-

fold within the skeletal (Gaitanos et al. 1993). The final release of chemical energy to virtually all biological processes in human cells and those in all other living organisms is the ATP molecule which is broken down into ADP (adenosine diphosphate) and free phosphate. During short and intense exercise, the first source of energy for contraction comes directly from stored ATP being hydrolyzed. Concurrently, chemical energy from creatine phosphate is transferred to form new ATP molecules. For work performed up to 90 seconds, anaerobic lactate formation provides most of the ATP needed. Over longer periods of work, ATP is primarily produced by oxidative phosphorylation in the mitochondria, which are highly abundant in skeletal muscle fibers, especially in type I fibers (the oxidative fibers). The body uses as much ATP in kilo gram as your body weight over a day (Törnroth-Horsefield & Neutze 2008) which means that the recycling rate is highly efficient. To produce ATP through aerobic metabolism, the muscle needs oxygen and nutrients (glucose and fatty acids) supplied via a network of capillaries interspersed in the fiber bundles (Windhorst & Mommaerts 1996).

1.2.2 Response to physical activity

Skeletal muscle is highly adaptable and responds to various external and internal stimuli such as exercise. Both acutely and over time, skeletal muscle demonstrates remarkable plasticity in structural and functional modifications and remodels itself in response to contractile activity and increased load. Metabolic adaptations in the different types of skeletal fibers, improved metabolic and mitochondrial functions as well as increased mitochondrial density and angiogenesis are some of the changes that occur in the skeletal muscle in response to the increased energy and structural demands that regular exercise induce (Egan & Zierath 2013).

Exercise disrupts the resting skeletal muscle homeostasis and is dependent on the exercise type performed (aerobic or anaerobic) and the type of contraction conducted (concentric, eccentric or isometric or combination thereof). This disruption leads to muscle adaptation, and the frequency, intensity and duration, *i.e* the dosage, of the exercise performed will also affect this process. The metabolic and molecular responses might differ dependent on the exercise types, and usually reflects a certain functional outcome (*e.g.* endurance versus hypertrophy phenotype) (Coffey et al. 2006; Coffey & Hawley 2007). Although both aerobic and resistance exercise can individually promote substantial health benefits, different effects are observed depending on the parameter of interest. Even though both types of exercise improve glucose control and lower resting blood pressure, aerobic exercise training more

effectively modifies cardiovascular risk factors and resistance exercise training more effectively increase basal metabolic rate, muscle mass, and physical function in the elderly (Booth & Thomason 1991). Importantly, a combination of aerobic and resistance training is more effective than either modality alone for reducing insulin resistance and functional limitations in patients with obesity or the metabolic syndrome (Davidson et al. 2009; Colberg et al. 2010).

1.2.3 PGC-1 α and exercise induced transcriptional regulation

Peroxisome proliferator-activated receptor (PPAR)- γ coactivator (PGC)-1 α , (PGC-1 α), is a transcription coactivator encoded from the PPARGC1A gene located in chromosome 4 and codes for a 798 amino acid protein (Liang & Ward 2006).

Both acute and regular exercise have long been known to increase the gene expression and protein levels of PGC-1 α both in skeletal muscle and in other metabolically active tissues such as brain, kidney and fat (Holloszy & Booth 1976; Baar et al. 2002; Norrbom et al. 2004; Gibala et al. 2009; D. A. Hood 2009; Steiner et al. 2011; Norrbom et al. 2011; Gidlund et al. 2015). PGC-1 α is a very multi-faceted protein when it comes to function and action, playing different roles depending on the demands and needs of the cell. PGC-1 α have been labelled a “master regulator” of mitochondrial biogenesis and as an important factor controlling the transcriptional machinery in the cell in response to increased energy demands as seen by metabolic stress or exercise (Z. Wu et al. 1999; J. Lin et al. 2005). PGC-1 α is a coactivator, that coordinates expression of numerous nuclear-encoded mitochondrial transcription factors which have been proposed to be vital for the regulation of mitochondrial biogenesis (Z. Wu et al. 1999; J. Lin et al. 2005), see Fig. 2 for illustration. Some of the regulatory functions of the PGC-1 α protein appears to be mediated by its strong co-activation of the nuclear respiratory factors 1 (NRF-1). This co-activation has been shown to be involved in both regulation of mitochondrial biogenesis and fiber type determination in skeletal muscle (Virbasius & Scarpulla 1994).

Other important factors are mitochondrial transcription factor A (TFAM), mitochondrial transcription factor B1 and B2 (TFB1M, TFB2M) and the nuclear respiratory factor 2 (NRF-2). PGC-1 α has been shown to induce vascular endothelial growth factor (VEGF) gene expression by coactivation with estrogen-related receptor- α (ERR α) (Arany et al. 2008). And coactivation of PGC-1 α with NRF-1 and/or ERR α has also been shown to increase

transcriptional activity of genes coupled to oxidative phosphorylation such as succinate dehydrogenase (SDH), β -oxidation and replication of the mitochondrial mtDNA (Kelly & Scarpulla 2004; Scarpulla 2011).

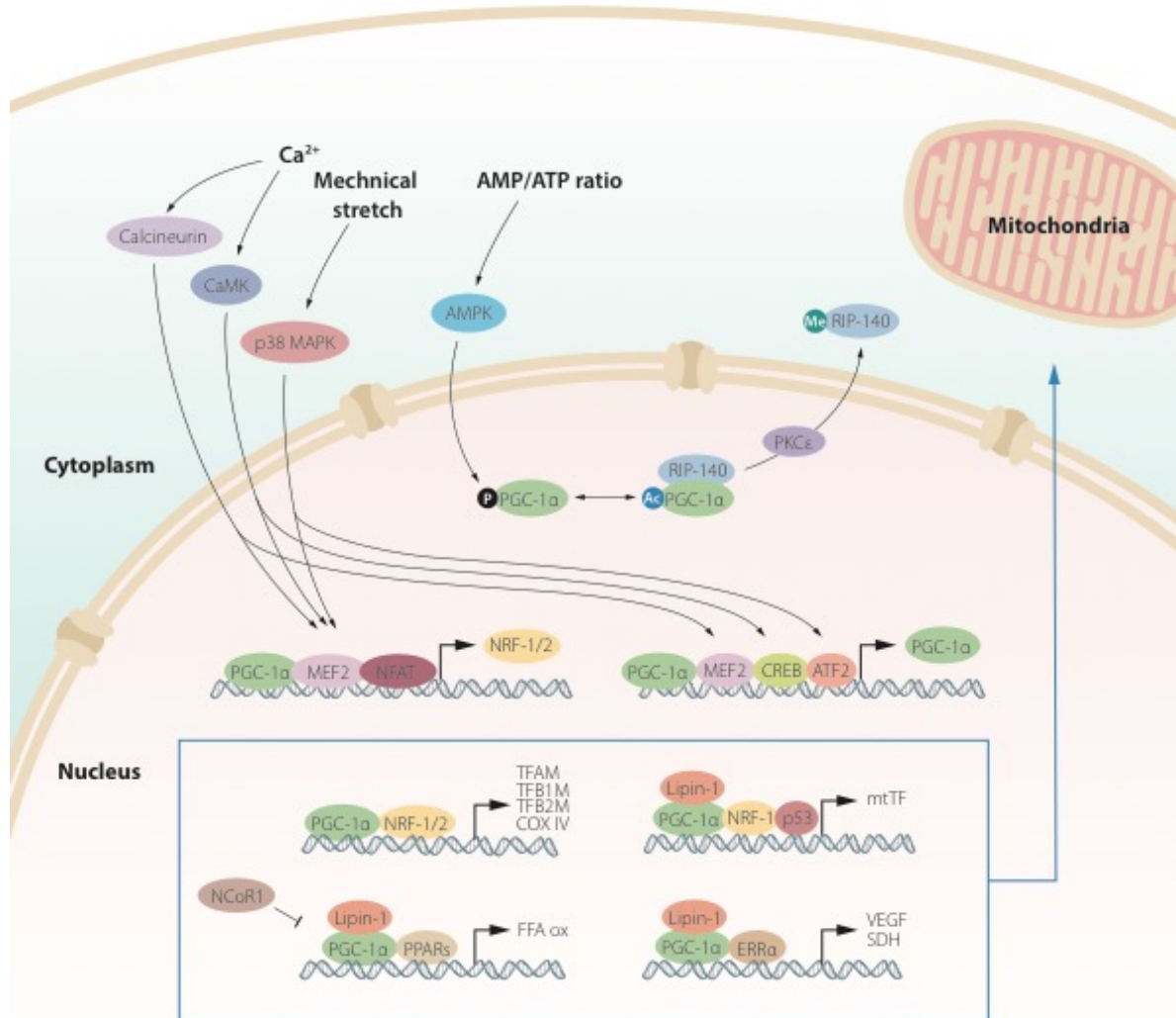


Figure 2. Schematic illustration of a human skeletal muscle cell and its nucleus showing factors involved in exercise-induced adaptations. During and following exercise, stimuli such as mechanical stretch, calcium signaling and AMP/ATP ratio are affected (displayed in the cytoplasm). Effects of exercise on transcriptional regulation are shown inside the nucleus. The box shows example of nuclear-encoded mitochondrial transcriptions factors that are activated by PGC-1 α . *Illustration: Mattias Karlén*

Previous studies from our lab and others have shown that PGC-1 α gene and protein expressions are highly induced by exercise (Pilegaard et al. 2003; Norrbom et al. 2004; Norrbom et al. 2011; Gidlund et al. 2015). The network that is activated by PGC-1 α has been shown to be downregulated by the nuclear receptor interacting protein-1 (RIP140). Previous studies have shown a connection between RIP140 and PGC-1 α such that transcription factors activated by PGC-1 α , *e.g.* NRF-1, are repressed by RIP140 (Hallberg et al. 2008). Also, strengthening the tentative metabolic role of RIP140 is the fact that the absence of RIP140 leads to resistance to diet-induced obesity, and increased glucose clearance and insulin sensitivity in mice (Powelka 2005; Parker et al. 2006). It has also been shown that RIP140 is present in muscle tissue in a fiber specific manner, and that high expression of RIP140 suppresses the formation of oxidative fibers (Seth et al. 2007). Nevertheless, lower levels of RIP140 do not seem required for exercise mediated increase of skeletal muscle mitochondrial content and the inhibition of metabolic target genes driven by RIP140 might be bypassed by exercise (M. S. Hood et al. 2011; Frier et al. 2011; Hoshino et al. 2011; Gidlund et al. 2015).

Taken together, PGC-1 α coordinates a broad metabolic and transcriptional network regulating oxidative metabolism and insulin sensitivity. This is essentially the same metabolic program that is activated by exercise and has been shown to be down-regulated by sedentary lifestyles as well as obesity and T2DM (Benton et al. 2008).

1.2.4 Glucose metabolism and regulation

The metabolic pathway of glucose breakdown in mammalian cells is called glycolysis, which is a chain reaction where glucose-6-phosphate is broken down to two pyruvic acids. The net products produced in the glycolysis are two ATP molecules, two molecules of the reduced form of the nicotinamide adenine dinucleotide (NADH) and two H^+ . The pyruvic acids are transformed into acetyl coenzyme A (acetyl-CoA) in the mitochondria. This molecule, Acetyl-CoA, is then used to produce the energy-rich compound (GTP), CO_2 , NADH and the reduced form of flavin adenine dinucleotide ($FADH_2$) in the citrate acid cycle (tricarboxylic acid (TCA) cycle) in the mitochondrial matrix. NADH and $FADH_2$ (usually referred to as electron carriers) are oxidized in the electron transport chain (ETC), a process also called oxidative phosphorylation that is located in the mitochondrial membranes (Ramaiah 1976).

During exercise, the main substrates for skeletal muscle contraction come from muscle glycogen and blood glucose (derived from liver glycogenolysis and gluconeogenesis) as well as from ingested carbohydrates. The regulation of muscle glycogenolysis during exercise is dependent on both intramuscular factors and hormonal stimulation of the enzyme glycogen phosphorylase. Insulin is a major regulator of glucose uptake and by binding to and phosphorylating the insulin receptor at the cellular surface, an intracellular cascade is activated that leads to glucose uptake into the skeletal muscle cell. This intracellular cascade includes the insulin receptor substrate 1 (IRS-1) which binds to the phosphorylated tyrosine residues of the insulin receptor and is subsequently phosphorylated by the tyrosine kinase of the insulin receptor. Binding of IRS-1 to the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) results in activation of a PI3-K-dependent pathway comprising phosphoinositide-dependent kinase (PDK) and protein kinase C (PKC) (Röhling et al. 2016). Key downstream molecules modulate translocation of glucose transporter type 4 (GLUT4) to the plasma membrane and comprise, besides Akt/protein kinase B (Akt/PKB), also Ras-related C3 botulinum toxin substrate 1 (Rac1), the TBC1 domain family member 1 (TBC1D1), or the Akt substrate of 160 kDa (AS160) (Röhling et al. 2016). However, skeletal muscle contraction by itself has been shown to translocate GLUT4 in an insulin-independent manner (Hayashi et al. 1997). The clearance of glucose due to muscle contraction has been shown to be a more potent stimulus than maximal insulin-stimulation (James et al. 1985).

The glucose uptake in skeletal muscle during exercise occurs by facilitated diffusion. Glucose uptake is affected by the amount of glucose delivered, the sarcolemmal glucose transport and the intracellular clearance of glucose. During exercise bouts the glucose uptake by the

skeletal muscle can increase up to 50-fold (Syrow et al. 2017). This insulin-independent glucose uptake have been shown to involve regulatory factors such as the activation of the AMP-dependent protein kinase (AMPK), increased sarcoplasmic Ca^{2+} that may involve Ca^{2+} -calmodulin-dependent protein kinase (CaMK) and PKC, nitric oxide (NO), and reactive oxygen species (ROS) (E. A. Richter & Hargreaves 2013). Redistribution of the blood flow to working skeletal muscles during exercise enhances glucose delivery to, and glucose uptake by, the contracting skeletal muscle. Increased plasma glucose levels, *e.g.* following carbohydrate ingestion, has been shown to increase muscle glucose uptake during exercise (McConell et al. 2000). During exercise and contraction, GLUT4 is translocated very rapidly to the cell membrane and cannot be seen as a limiting factor for muscle glucose uptake. Increased exercise intensities also increase the glucose uptake in an intensity- and duration dependent manner (E. A. Richter & Hargreaves 2013). As a response of the muscle contraction, activation of the glycolytic and oxidative pathways within the muscle leads to increased glucose metabolism and maintained low intracellular glucose concentration as well as ATP production. Exercise training can increase the efficiency of the skeletal muscle to metabolize glucose (translocation of GLUT4 and enzymatic upregulation, especially in the glycolysis) and is an essential component in the prevention and treatment strategy for glucose-related metabolic diseases such as T2DM.

1.2.5 Fat metabolism and regulation

The breakdown of fatty acid is called β -oxidation and occurs within the mitochondria. β -oxidation is a process consisting of multiple steps by which fatty acids are broken down to acetyl-CoA and produces NADH and FADH_2 . As described above, acetyl-CoA is used in the citric acid cycle and NADH and FADH_2 are mainly used in the ETC. Thus, to be able to enter the cell, fatty acids need specific transporters. Fatty acid transporters include fatty acid translocase (FAT/CD36), tissue specific fatty acid transport proteins (FATPs), and plasma membrane bound fatty acid binding protein (FABP) (Lopaschuk et al. 2010; Holloway et al. 2011). Once inside the cell, a CoA group is added to the fatty acid by fatty acyl-CoA synthase (FACS), forming long-chain acyl-CoA. To enter the mitochondria carnitine palmitoyltransferase 1 (CPT1) converts the long-chain acyl-CoA to long-chain acylcarnitine which allows the fatty acid to be transported across the inner mitochondrial membrane via carnitine translocase (CAT). This transport is a recycling process which exchange long-chain acylcarnitines for carnitine. Carnitine palmitoyltransferase 2 (CPT-2) located in the inner

mitochondrial membrane then converts the long-chain acylcarnitine back to long-chain acyl-CoA, see Fig. 3. The long-chain acyl-CoA enters the fatty acid β -oxidation pathway, which results in the production of one acetyl-CoA from each cycle of fatty acid β -oxidation. If the fatty acid contain an odd number of carbons it is oxidized to one acetyl-CoA and one propionyl-CoA in the last cycle step of β -oxidation (Fukao et al. 2004).

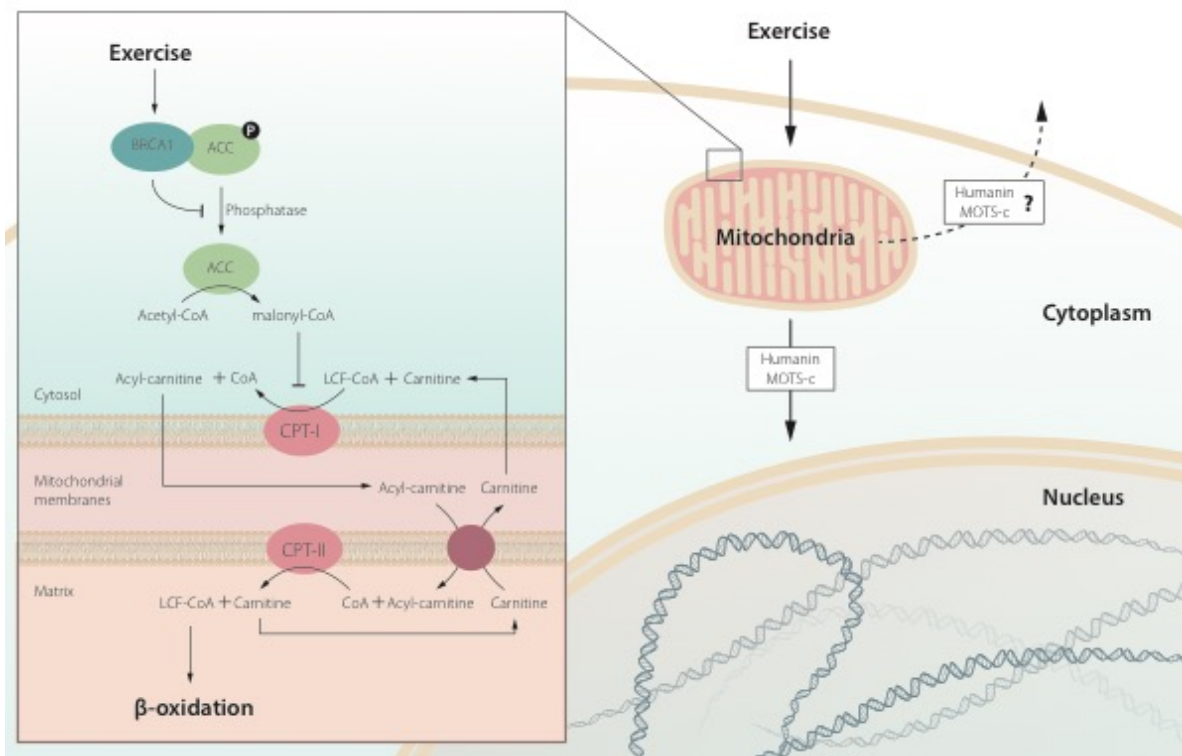


Figure 3. Schematic illustration of a human skeletal muscle cell and the action of the BRCA1 protein. The figure illustrates the nucleus (bottom right) and shows factors involved in transportation of fatty acids over the mitochondrial membranes (CPT-I and CPT-2). Retrograde signaling by humanin and MOTS-c is also displayed. *Illustration: Mattias Karlén*

Fat oxidation contributes significantly to whole-body energy turnover both at rest and during submaximal exercise. During exercise and upon muscle contraction, FAT/CD36 moves to the plasma membrane and increases fatty acid uptake. An increased oxidation of fatty acids have been shown to delay the depletion of carbohydrates and will, therefore, improve endurance during prolonged exercise (Sahlin et al. 2008). An efficient fat oxidation is not only of importance for exercise performance but also an health benefits in reducing the risk of developing metabolic diseases, as discussed in section 1.1. Acetyl-CoA carboxylase (ACC) is a central enzyme involved in fatty acid β -oxidation and fatty acid biosynthesis. ACC

catalyzes the carboxylation of acetyl-CoA producing malonyl-CoA (MaCoA). MaCoA inhibits mitochondrial fatty acid uptake by inhibiting the action of CPT1 (Lopaschuk et al. 2010). The main pathway for the degradation of MaCoA is via malonyl CoA decarboxylase (MCD), which decarboxylates malonyl CoA to acetyl CoA and thereby hinder its inhibitory activity.

Another way to modulate MaCoA activity has been suggested to involve the breast cancer 1 early onset (BRCA1) protein. BRCA1 is a pleiotropic and estrogen-sensitive gene, mainly coupled to DNA repair and involved in tumor suppression. However, BRCA1 has also been shown to interact with the phosphorylated form of acetyl CoA carboxylase (ACC-p) and thereby decreasing the inhibitory action of MaCoA in human breast cancer cells (Magnard et al. 2002; Moreau et al. 2006).

Fatty acid oxidation might also be regulated transcriptionally by factors regulating the expression of genes coding for proteins involved in fatty acid uptake and oxidation, as well as specific enzymes involved in the β -oxidation. Such transcriptional control is exerted by the relatively large family of nuclear receptors called PPARs (PPAR- α , PPAR- δ , and PPAR- γ) which are subject to transcriptional coactivation by PGC-1 α (Lopaschuk et al. 2010). Genes coding for *e.g.* FAT/CD36 and CPT1 have been shown to be regulated by PPAR- α (Yang & Li 2007). To summarize, the regulatory mechanism for fatty acid oxidation can occur in several different steps within the process. Numerous factors seem to affect the fatty acid oxidation, including *e.g.* exercise (in an intensity and duration dependent way), BRCA1 through the interaction with ACC-p and PGC-1 α by regulating transcription of genes important for β -oxidation and mitochondrial biogenesis.

1.3 THE MITOCHONDRIA

1.3.1 The magic of the mitochondria

Billion years ago, free-living aerobic bacteria (a prokaryote) managed to survive the endocytotic engulfment by a eukaryotic cell and a successful symbiosis was established. This is called the endosymbiotic theory which lead to one of the most enduring symbiotic relationships known in biology (Stewart & Chinnery 2015). This symbiotic relationship formed the organelle that we call the mitochondria and made the evolution of more complex multicellular organisms possible (Gray et al. 1999) .

This symbiosis occurred around at the same time as oxygen tension in the earth's atmosphere began to rise (Farquhar et al. 2000; E. O. L. Karlberg & Andersson 2003) which provided a survival benefit for the eukaryotic cell since an oxygen-dependent highly efficient metabolic system became available (Gray et al. 1999). This oxygen-dependent system in the mitochondria is essential for ATP production by oxidative phosphorylation. Without the fusion of the eukaryotic cell and these bacteria all living animals would be dependent on anaerobic glycolysis for ATP production. In addition, the mitochondria are abundantly present and vital for animal cells, and involved in lipid and amino acid metabolism and play important roles in various cellular processes such as cell proliferation, apoptosis and cell differentiation (M. Sato & K. Sato 2013).

1.3.2 Function, structure and biogenesis

1.3.2.1 The genome and proteome of the mitochondria

Mitochondria are organelles surrounded by two membranes, the outer and the inner membrane. This membrane structure forms two separate aqueous compartments, the matrix and the intermembrane space (see Fig. 4). The cristae of the mitochondria are formed by tubular invaginations of the inner mitochondrial membrane, in which the enzyme complexes of the ETC are abundantly located and those providing the cell with energy in the form of ATP. Located within the matrix of the mitochondria the other metabolic systems involved in glucose and fatty acid breakdown, the TCA cycle and the β -oxidation, can be found (Falkenberg et al. 2007).

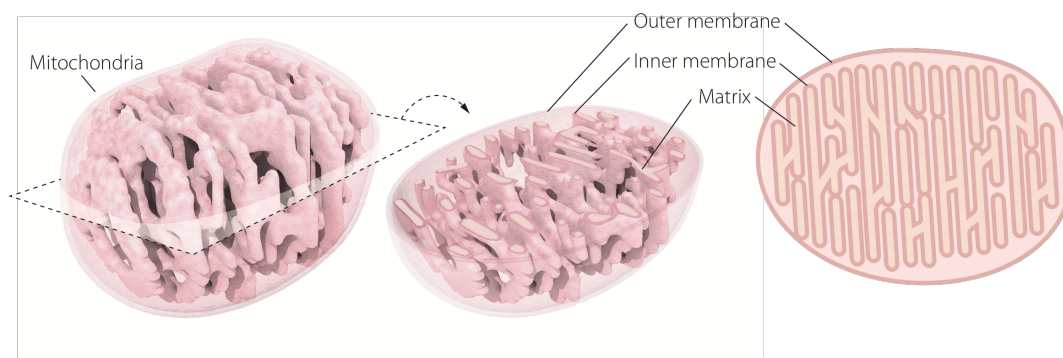


Figure 4. Illustrations of mitochondrial structure. *Illustration: Mattias Karlén*

Even though the mitochondria are enclosed within a membrane and defined as an organelle, they should not be considered as single entities, rather a network of interconnected membranes making up a tubular dynamic reticulum within the cell (Kirkwood et al. 1986; Sukhorukov et al. 2012). Fusion and fission dynamics are constant ongoing events of the mitochondria which leads to branching of the reticulum of tubules (Sukhorukov et al. 2012).

Mitochondria are unique organelles since they contain their own circular DNA (mitochondrial DNA, mtDNA). DNA of this cytoplasmic organelle, the mitochondria, is not inherited in a Mendelian manner. It is widely accepted that mtDNA is inherited maternally, solely from the mitochondria of the oocyte from which the animal develops (M. Sato & K. Sato 2013). Human mitochondria contain a compact circular, double-stranded molecule of 16 569 bp (16.6 kbp) genome (see Fig. 5), with no known introns and very few non-coding nucleotides. Traditionally, the human mtDNA has been considered to contain 37 genes, coding for two rRNAs, 22 tRNAs and 13 polypeptides (Falkenberg et al. 2007). The small size of the mtDNA limits its coding capacity and is thought only to account for a small fraction of the organelle's entire proteome, which consists of at least 1500 different proteins. The 13 proteins encoded by mammalian mtDNA are all components of the ETC. Different versions of the endosymbiotic theory have argued that there was a massive transfer of genes from the endosymbiont into the nuclear genome during the evolution of the mitochondrion. Indeed almost all of the genes encoding the proteins of modern mitochondria are found in the nuclear genomes of their host cell (O. Karlberg et al. 2000). Thus, the mitochondrial genomic machinery does not unaided control the organelle's proteome. The remaining ~77 subunits

involved in the ETC are encoded by nuclear genes, as are all proteins required for the transcription, translation, modification, and assembly of the 13 mtDNA proteins (Calvo & Mootha 2010). However, this view has recently been somewhat challenged and previously unknown features of mitochondrial gene expression, function and regulation have been suggested which indicate that the mitochondrial transcriptome and proteome are far more complex than previously thought (Hashimoto, Ito, et al. 2001; Mercer et al. 2011).

Nuclear genome insertions of mitochondrial origin known as NUMTs have also been identified (Bensasson et al. 2001; Ramos et al. 2011). In 1967 the first report of DNA fragments with homology to the mitochondrial genome was published (Buy & Riley 1967). Later, the nuclear mitochondrial pseudogenes arose as a concept. A possible explanation for these integrations of mtDNA is incorporation into the nuclear genome during the repair of chromosomal breaks by nonhomologous recombination. Such hypothesis, of a possible incorporation of mtDNA, is supported by the presence of mtDNA fragments in the nucleus (Bensasson et al. 2001; Mishmar et al. 2004). There are over 500 NUMTs in the human genome (Mishmar et al. 2004; Richly & Leister 2004). Even though most NUMTs are considered pseudogenes, bioinformatics based evidence suggests that at least some of the nuclear sequences might be functional genes (Bodzioch et al. 2009).

To summarize, the mitochondria are unusual and vital organelles, surrounded by two membranes, contain its own circular DNA and make up a dynamic network which acts as the powerhouse of the cell.

1.3.2.2 Transcription and replication of the mitochondrial DNA

Contrary to the nuclear genome, mitochondria are continuously turned over and replicated independent of the cell cycle (Bogenhagen & Clayton 1977). The mitochondrial chromosome contains no introns. There is, however, a non-coding regulatory region known as the displacement loop (D-loop) in which the promoter for transcription of both the heavy strand (HS1) and the light strand (LS) are located (Montoya et al. 1982). Almost the entire heavy strand is transcribed from the other heavy strand (HS2) promoter (located in proximity to the D-loop) and the entire light strand is transcribed from the LS promoter (Stewart & Chinnery 2015). The HS1 promoter initiate the transcription of the two mitochondrial rRNA molecules (Clayton 2000b; Stewart & Chinnery 2015), see Fig. 5.

Transcription from the mitochondrial promoters results in polycistronic precursor RNA molecules, that are processed to yield individual mRNA, rRNA and tRNA molecules (Falkenberg et al. 2007). Replication and transcription of mtDNA are tightly coupled, with LS transcription producing RNA primers for mtDNA replication initiation (Clayton 2000b). Although the mitochondria are self-sufficient when it comes to the production of ribosomal subunits and tRNA molecules, enzymes and other factors required for transcription of mtDNA are nuclear-encoded and subsequently imported to the mitochondrial matrix.

Figure 5. Schematic illustration of the mitochondrial DNA molecule. Shown are the heavy strand, the light strand, the light strand promoter (LSP), the heavy strand promoter 1 and 2 (HSP1, HSP2), the D-loop as well as the origin of light strand replication site (OL) and the origin of heavy strand replication site (OH). Redrawn from the book - Abdul Aziz Mohamed Yusoff, F.A.Z.I.H.J. & Abdullah, J.M., 2015. "Understanding Mitochondrial DNA in Brain Tumorigenesis. In Molecular Considerations and Evolving Surgical Management Issues in the Treatment of Patients with a Brain Tumor". InTech.

Illustration: Eva-karin Gidlund

Precursors of nuclear-encoded mitochondrial proteins are transported over the mitochondrial membranes by specific transport complexes, the translocase of the outer membrane (TOM) and the translocase of the inner membrane (TIM) (Dudek et al. 2013). Mitochondrial transcription requires nuclear-encoded protein such as mitochondrial RNA polymerase (POLRMT) with assistance and co-activation of the mitochondrial transcription factors, TFAM, together with either TFB1M or TFB2M. The genes encoding TFB1M and TFB2M

are ubiquitously expressed with the highest mRNA levels detected in heart, skeletal muscle and liver and both TFB1M and TFB2M can form a heterodimeric complex with POLRMT (Asin-Cayuela & C. M. Gustafsson 2007). However, how the mammalian mitochondrial transcription machinery recognizes promoter sequences is not yet fully understood. POLRMT in complex with TFB1M or TFB2M cannot initiate transcription in the absence of TFAM. One possible role for TFAM might be to introduce specific structural alterations in mtDNA, for example, unwinding of the promoter region, which might facilitate transcription initiation (Asin-Cayuela & C. M. Gustafsson 2007; Sologub et al. 2009). TFAM have also been shown to be upregulated in expression by NRF-1, which coordinates nuclear encoded respiratory chain expression with mitochondria gene transcription and replication. Moreover, mitochondrial transcription termination factors (MTERFs) have also been described as a family of additional regulators displaying multiple roles in the regulation of mitochondrial transcription (Roberti et al. 2009).

Recently, TFAM has also been suggested to play a role in the replication and checkpoint system of mtDNA (Lyonnais et al. 2017). Replication of the mtDNA is necessary for maintenance of the organelle and for mitochondrial biogenesis to occur (Medeiros 2008). The replication of mtDNA, is also highly dependent on nuclear events. The proteins known to be of importance for this process are DNA polymerase γ (POLG), mitochondrial single-stranded DNA-binding protein (mtSSB) and the Twinkle helicase (also known as PEO1). The Twinkle helicase as the ability to unwind short segments of the mtDNA and thereby aiding the replication process (Wanrooij & Falkenberg 2010). Unlike nuclear DNA, which is packaged into nucleosomes, mtDNA molecules are tightly associated with the mitochondrial matrix and form compact structures called nucleoids, composed of mtDNA-protein complexes that include proteins involved in replication and transcription such as mtSSB, DNA POLG, and TFAM (Spelbrink 2010). The RNA primers used to initiate mtDNA synthesis at the origin of replication for the heavy strand (HS) called the OH site (also known as OriH), are generated from mitochondrial RNA (Clayton 2000a). Copying of the heavy strand later facilitates priming of replication of the origin of light strand replication site (OL).

Two models of mtDNA replication have been proposed, the strand-displacement model and the symmetric strand-coupled replication (Shadel & Clayton 1997). Mammalian mtDNA molecules replicate by the strand-displacement model and replication is induced by transcription within the non-coding D-loop. In brief, the replication proceeds clockwise from the OH until the OL is exposed, allowing light strand synthesis to proceed clockwise until the entire molecule is copied.

When the mitochondrial replisome responsible for replication proceeds clockwise past the D-loop region, two thirds of the growing HS is formed before a point is reached at which growing LS synthesis can start at OL circle (Clayton 2000a; Stewart & Chinnery 2015). As a newly exposed single-stranded template sequence in the HS forms a hairpin to constitute OL, HS replication (into an emerging LS) commences in the opposite direction. Both strands are thus replicated as leading strands (5'→3' directed) rather than lagging strands (Abdul Aziz Mohamed Yusoff & Abdullah 2015). The progeny molecules are released as dissimilar free circles. The new double-stranded mtDNA molecule is formed through the removal of the RNA primers, gap-filling, introduction of super-helical turns and closure of the circle (Clayton 2000a; Stewart & Chinnery 2015).

In addition, POLRMT and the transcriptional machinery mentioned above also influences the replication process of mtDNA. POLRMT generates the RNA primers used to initiate leading-strand mtDNA synthesis at the origin of heavy strand DNA replication (FustE et al. 2010). The transcription factor PGC-1 α is also an important regulator of mitochondrial biogenesis by its strong co-activation of NRF-1. In turn, NRF-1 activates TFAM and TFB1M and TFB2M and thereby stimulates the cell to increase its mitochondrial copy number (Fisher et al. 1992; Falkenberg et al. 2002), which illustrates that mitochondrial replication and transcription are tightly linked (Holt & Reyes 2012).

In summary, the morphology and functional properties of mitochondria are, under a highly-regulated fashion, finely tuned to meet changes in energetic, metabolic, and signaling demands of the skeletal muscle cell.

1.3.3 Retrograde signaling

The symbiosis between the mitochondrion and the eukaryotic cell created the need for a communication system between mitochondria and the nucleus to coordinate mitochondrial protein synthesis during biogenesis and to communicate possible mitochondrial malfunctions. Communication from the mitochondria to the nucleus is referred to as retrograde signaling, and communication from the nucleus to the mitochondria is known as antegrade signaling (da Cunha et al. 2015). The mitochondria have traditionally been perceived as end-function organelles that receive cellular signals and regulate processes such as energy conversion and apoptosis in response to these signals. However, in recent years it has become evident that cellular homeostasis requires a constant and active flow of information between the mitochondria and the nucleus (C. Lee et al. 2013). The research has mainly been focused on a limited number of retrograde signaling molecules and signaling pathways such as sirtuins, cytochrome C, ROS, Ca^{2+} , Fe^{2+} , NO and CO (Verdin et al. 2010; Ganta & Alexander 2009; Ichikawa et al. 2012). It has also been shown that mtDNA itself can act as a retrograde signal in the inflammation response or by other cellular stress (Nakahira et al. 2011; Saki & Prakash 2016). In addition to subcellular signaling programs, mitochondrial factors can even be released from one cell and exert paracrine or endocrine effects on a different cell, *e.g.* cellular stress can result in externalization of bits of mtDNA, and this circulating mtDNA have been shown to evoke a systemic inflammation response (Q. Zhang et al. 2010). Beyond these retrograde signals, recent studies have identified a host of mitochondria-linked factors that influence the cellular and extracellular environments, including mitochondria-derived peptides (MDP's) and mitochondria-localized proteins (Battersby & U. Richter 2013; C. Lee et al. 2013; Kadlec et al. 2016). The first described mitochondria-derived peptide was humanin (HN) (Hashimoto, Niikura, et al. 2001), and more recently another small peptide also encoded from the mtDNA called MOTS-c was discovered (C. Lee et al. 2015), see Fig. 3. Moreover, in 2013 transcriptional profiling identified over 70 different transcription factors that actively were involved in mitochondrial retrograde signaling and among those PGC-1 α and hypoxia inducible factor (HIF-1) were presented as some of the main candidates affecting retrograde signaling pathways (Chae et al. 2013).

1.3.4 A gene within a gene

In 2001, the mitochondrially-derived peptide humanin (HN) was identified from a cDNA library from the surviving neurons of human Alzheimer's disease brain (Hashimoto, Niikura, et al. 2001). This 24-amino acid polypeptide was shown to be transcribed from a 75 bp open reading frame (ORF) of the 16S rRNA (MT-RNR2) gene within the mtDNA which makes it a gene-within-a gene. The term humanin was coined by its discoverer, Professor Nishimoto, to denote the potential of this molecule to restore the 'humanity' of patients with Alzheimer's disease since HN previously was described as a neuroprotective and anti-apoptotic factor (C. Lee et al. 2013). Whether HN is translated in the mitochondrion or the cytoplasm is not fully known. Mitochondrial HN translation result in a slightly shorter peptide (21 amino acids) than that of cytoplasmic translation (24 amino acids) (Yamagishi et al. 2003; Bin Guo et al. 2003). Both the HN peptide originating from the mitochondria and cytoplasm has been shown to be biologically active but the translational site in humans remains undetermined (Bin Guo et al. 2003; Sreekumar et al. 2016). HN has been shown to interact with the insulin-like growth factor binding protein 3 (IGFBP- 3) (Ikonen et al. 2003) and the pro-apoptotic protein BAX (Bin Guo et al. 2003). HN could be an important link for understanding the role of the mitochondria beyond its central function as a cellular "power house". The role and function of HN is complex and several other features have been coupled to HN activation, such as improved beta cell function and peripheral insulin signaling (Muzumdar et al. 2009). The HN analog HNGF6A has also been shown to increase glucose-stimulated insulin secretion in both normal and diabetic mice (Kuliawat et al. 2013). Interestingly, HN levels in plasma have recently been demonstrated to be lower in patients with impaired fasting glucose compared to a control group (Voigt & Jelinek 2016). The involvement of HN in mitochondrial function was recently shown in human retinal pigment epithelial cells treated with HN *in vitro*, which displayed marked increase in mtDNA copy number and upregulated TFAM protein (Sreekumar et al. 2016).

The initial discovery of HN spurred the search for more MDP's and in 2015 a small 16-amino acid peptide encoded within an ORF (51 bp) of the 12S rRNA gene (MT-RNR1) called MOTS-c (mitochondrial open reading frame of the 12S rRNA-c) was discovered (C. Lee et al. 2015). MOTS-c has been shown to be important for glucose regulation by inhibiting the folate cycle and its tethered *de novo* purine biosynthesis, leading to AMPK activation in skeletal muscle. Also, levels of MOTS-c are reduced in obese people and patients with insulin resistance (C. Lee et al. 2015). Like a Russian nesting doll, HN and MOTS-c are

genes-within-a-gene, within an organelle within a cell, and the function of these MDP's are still not fully understood.

1.3.5 The mitochondria, impaired glucose regulation and T2DM

Defects in mitochondrial function have been linked to many of the most common diseases of aging such as T2DM, Parkinson's disease, atherosclerotic heart disease, stroke, Alzheimer's disease, and cancer. Mitochondrial dysfunction has been associated with changes in mRNA levels of mitochondrial markers, alterations in protein levels or in enzymatic activity of key components of the mitochondrial oxidative machinery as well as changes in mitochondrial size, shape and substrate oxidation (Montgomery & Turner 2015). In brief, studies have shown that genes involved in the mitochondrial oxidative phosphorylation are down-regulated in muscle of insulin-resistant patients suffering from obesity or T2DM (Kelley et al. 2002; Patti et al. 2003; Ritov et al. 2005). It has also been shown that obese people have a reduced whole-muscle mitochondrial content which has been suggested to be a result of impaired mitochondrial biogenesis (Kelley et al. 2002; Holloway 2009). However, human exercise studies have demonstrated a clear connection between exercise and improvements in mitochondrial function in T2DM patients (Rimbert et al. 2004; Toledo et al. 2007; Phielix et al. 2010; Nielsen et al. 2010; Meex et al. 2010). The action by which the mitochondria might be an important player in the restoration and prevention of IGR and T2DM seems to be connected to PGC-1 α abundance and activity, and it also seems like this is the pathway activated by exercise (Patti et al. 2003; Mootha et al. 2003; Short et al. 2003).

2 AIMS

Understanding fundamental molecular mechanisms behind skeletal muscle adaptations to exercise and regular physical activity has high scientific importance. Additionally, insights into such mechanisms can form the basis for preventing and treating metabolic and chronic diseases linked to physical inactivity, ageing and obesity.

The overall aim of this thesis was to examine PGC-1 α and its surrounding network and related regulatory factors important for skeletal muscle adaptation to physical activity. The main focus was to improve the understanding of the molecular mechanisms that contribute to mitochondrial adaptations both in healthy humans and in men with impaired glucose regulation.

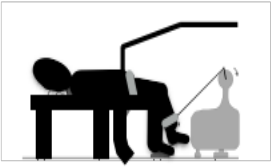
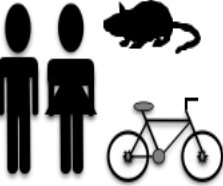
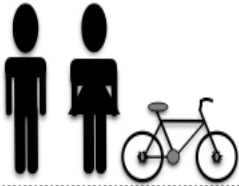

The specific aims of this thesis were to investigate;

- if metabolic perturbation affects the PGC-1 α transcripts in different ways (study 1).
- if exercise increases the expression of the PGC-1 α -ex1b isoform to a greater extent than that of PGC-1 α -ex1a (study 1 and 3).
- if BRCA1 is present in skeletal muscle and if so, whether it is involved in the regulation of metabolism in human and mouse skeletal muscle (study 2).
- the effect of a single exercise bout on the levels of PGC-1 α , its different transcripts and related regulatory factors over a 24-hour time course (study 3).
- if 12 weeks of either resistance or endurance-type training in men with IGR changes the expression of the mitochondrially encoded peptide humanin (study 4).

3 METHODOLOGY

3.1 SUBJECT CHARACTERIZATION AND INTERVENTION SUMMARY

Table 1. Schematic summary of included studies, material, interventions and paper of this thesis.

	Study 1	Study 2	Study 3	Study 4
	Acute - blood flow restriction	Acute + Mice	Acute - time course	12 weeks
	paper I	paper II	paper III	paper IV and preliminary results
				
Subjects in total	♂ n= 12	♂ n= 13 ♀ n= 13 ♂ C57B1/6 n= 20 ♀ C57B1/6 n= 14	♂ n= 10 ♀ n= 10	♂ n= 55
Intervention	Exercise with restricted blood flow and Exercise with nonrestricted blood flow	Human: Exercise Animal♦: Exercise and Control Animal♦: HFD and NCD	Exercise or Control	Resistance exercise, Nordic walking or Control
Biopsies	Pre, 0 min and 2-hrs post	Human: Pre and 30 min post Animal♦: 0 min post Animal♦: 10 weeks post	Pre, 30 min, 2h, 6h and 24-hrs post	Pre and 12 weeks post
Performed analyses	Real time RT-PCR, Immunoblotting	Real time RT-PCR, Immunoblotting, Immunoprecipitation, Nuc. and Cyt. fractions, Cell Culture, Adenovirus infection, Immunohistochemistry, Insulin Signaling, ROS measurement and imaging	Real time RT-PCR, Immunoblotting, Nuc. and Cyt. fractions	RT-PCR, ELISA, Enzyme activity (CS), RNAseq
Factors analyzed	PGC-1α tot, PGC-1α-ex1a, PGC-1α-ex1b, MCIP1 phospho-p38, total p38 phospho-AMPK, total AMPK	BRCA1 total, BRCA1Δ11, BRCA1Δ11b MaCoA phospho-ACC, total ACC phospho-Akt BRCA1-ACC-p ROS accumulation FFA accumulation	PGC-1α tot, PGC-1α-ex1a, PGC-1α-ex1b, non-trunc-PGC-1α, trunc-PGC-1α LIPIN-1, LIPIN-1α, LIPIN-1β ERRα, ERRγ, p53, NRF-1, TFAM, VEGF-A, VEGF-B, TFB1M, PKCε, SDHA, RIP140, NCoR1, NCoR1-1, NCoR1-2, NCoR1-3	Humanin, MT-RNR2, MT-RNR1, MT-RNR2-L1, MOTS-c, GLUT4, MEF2A, RIP140, TBC1D4, PGC-1α tot, PGC-1α ex 1b

3.2 EXPERIMENTAL MODELS AND TISSUE SAMPLING

3.2.1 Skeletal muscle biopsies and blood sampling in humans

Muscle biopsies, using the percutaneous muscle biopsy technique (Bergström 1962), were obtained from the *m. vastus lateralis* in all the human studies. Biopsies were taken before (all studies) and at different time points after either acute exercise or long-term training. Post biopsies were obtained at 0 min (**Paper I**), 30min (**Papers II and III**), 2h (**Papers I and III**), 6 hrs (**Paper III**), 24 hrs after an acute bout (**Paper III**) and 48-72 hrs (**Paper IV**) after the last training session in a 12-week intervention. Prior to the biopsy, the skin was shaved and cleaned with alcohol. Local anesthetic (carbocain 10 mg/ml) was injected in the skin and down to the muscle fascia. An incision was made through the skin and fascia. The biopsy needle was inserted through the incision into the muscle and a small biopsy was cut out. The biopsy was then snap frozen in liquid nitrogen and stored for further analyses. If more than one biopsy was obtained from the same muscle, a new incision was made approximately 2-4 cm proximal to the previous one. Blood samples were drawn after an overnight fast (12 h) from the brachial vein of all subjects before the intervention and after the 12-week intervention period (**Paper IV**). In this prolonged intervention study subjects were instructed to avoid alcohol, intensive exercise and analgesic for 2 days before sample collection. The amount of dietary carbohydrates was advised to match their normal consumption for 3 days before sample collection. Plasma, serum, and glucose tubes were centrifuged at 2200 g for 10 min in room temperature, plasma and serum samples were stored in multiple portions at -80 °C. Studies 1-3 was approved by the Regional Ethical Review Board in Stockholm, Sweden. All subjects gave their written informed consent before participating. The Ethic Committee of the Hospital District of Helsinki and Uusimaa in Finland approved the protocol of study 4 and all the study subjects gave written informed consent. All studies in this thesis conformed to the standards set by the Declaration of Helsinki. The University of Maryland IACUC Review Board approved all aspects involving animals both in the exercise study and of the high fat diet study (study 2).

3.2.2 Skeletal muscle sampling in mice

The University of Maryland Institutional Animal Care and Use Committee (IACUC) Review Board approved all aspects involving animal research. Male and female C57Bl/6 mice ranging from 8-10 weeks and 12-week-old male mice were utilized in **Paper II**. At the end of the exercise bout or after 10 weeks of a high fat diet (HFD) program, animals were euthanized and skeletal muscle (*m. tibialis anterior*, *m. soleus*, *m. gastrocnemius* (acute exercise study) and *m. plantaris* (HFD-study)) was harvested, weighted, snap frozen in liquid nitrogen, and stored at -80°C.

3.2.3 Training with restricted blood flow (Paper I)

Restricted blood flow has been shown to increase the energy demand and the intracellular exercise response within skeletal muscle cells, thereby mimicking extreme exercise intensity. In this study, twelve healthy men performed one-legged knee extension exercise using a pressure chamber model to induce blood flow restriction. This exercise model was first described back in 1987 (Eiken & Bjurstedt 1987) and further characterized by Sundberg and Kaijser (Sundberg & Kaijser 1992). External pressure over the working leg was used to restrict blood flow during exercise in a controlled fashion. The subjects mean (range) age, height, and weight were 24 (20-27) yrs, 181 (173-190) cm, and 75 (63-90) kg, respectively, and mean (range) maximal oxygen consumption ($\text{VO}_{2\text{max}}$) was 51 (43-64) $\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$. The subject was positioned supine in the opening of a large pressure chamber with both legs inside the chamber and one leg strapped to a pad. The pad was connected to a crank arm of an electrically braked cycle ergometer with locked flywheel. The chamber opening was sealed off at the level of the crotch by a rubber diaphragm. Shoulder supports were used to prevent cranial displacement of the body with increased chamber pressure. For the restricted (R) blood flow exercise, the chamber pressure was elevated to 50 mmHg above atmospheric pressure. This has been shown previously to restrict leg blood flow during one-legged cycle exercise by 15-20 %, reduce oxygen saturation by 10-12 percentage units (Sundberg & Kaijser 1992) and cause a greater depletion of ATP, creatine phosphate as well as increase lactate concentrations in the exercising leg (Sundberg 1994). In this sense, this type of exercise mimics the cellular response seen during and after extremely intense exercise. Exercise with non-restricted (NR) blood flow was performed using the same experimental set-up but with normal atmospheric pressure. The exercise bout consisted of 45 min with

restricted blood flow to the working leg, followed by 45 min with normal blood flow to the other leg. The subjects were randomized regarding which leg they should exercise in the R-condition. All subjects exercised the R-leg condition first, and were instructed to exercise at the highest tolerable workload for 45 min, taking into account that they must complete the entire session. After a short rest, exercise in the NR-leg condition was performed using the same workload profile as recorded for the R-leg condition but using normal atmospheric pressure. Accordingly, the two legs developed the same absolute power and amount of work in each session, although the ischemic training was perceived as much more strenuous. Muscle biopsies from the *m. vastus lateralis* of both legs were obtained using the percutaneous needle biopsy technique at rest before the exercise bout (R- and NR- condition) followed by post-exercise biopsies directly after and 2 hrs after exercise. The pre-biopsy in the NR-leg was performed at the same time as the 2 hrs post-biopsy in the R-leg. The subjects were resting between the biopsies.

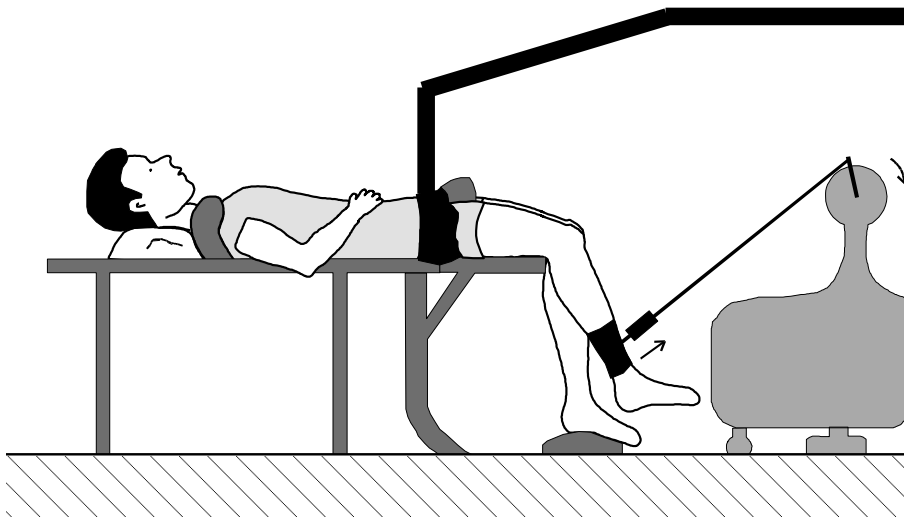


Figure 6. Schematic illustration of the experimental settings in study 1. One-legged knee-extension training performed in a pressure chamber. Modified from Ola Eiken (1987).

3.2.4 Acute exercise (Paper II and III)

To gain insight into the temporal resolution of gene expression after a single exercise bout, an acute exercise study was performed. Twenty-seven healthy subjects were included in the study. Out of these, ten men and ten women were enrolled in **Paper III** and thirteen men and thirteen women in **Paper II**. Some subjects were overlapping between study 2 and 3. Their mean \pm SD age, height, and weight, for men and women were; 26 ± 3.3 and 25 ± 2.9 yrs, 182 ± 6.2 and 169 ± 6.2 cm, and 81 ± 6.5 and 64 ± 6.5 kg in **Paper II** and 25 ± 2.7 and 24 ± 2.8 yrs, 182 ± 4.8 and 169 ± 6.5 cm and 80 ± 6.6 and 64 ± 6.5 kg in **Paper III** respectively. Prior to the intervention, their maximal oxygen uptake ($\text{VO}_{2\text{max}}$) was determined using an incremental cycle ergometer test until exhaustion. In **Paper II**, mean \pm SD $\text{VO}_{2\text{max}}$ was $50.9 \pm 4.9 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ for men and $43.8 \pm 7.1 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ for women. In **Paper III**, mean \pm SD peak maximal oxygen consumption ($\text{VO}_{2\text{peak}}$) was $50.2 \pm 4.6 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ for men and $41.5 \pm 3.6 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ for women. After inclusion in the study, subjects were randomly assigned to either an exercise group (ExG), or a non-exercising control group (CG). There were no significant differences between the groups regarding age, height, weight, and $\text{VO}_{2\text{peak}}$. The ExG performed 60 min of cycling exercise at a workload corresponding to; 50 % of $\text{VO}_{2\text{peak}}$ the first 20 min and 65 % of $\text{VO}_{2\text{peak}}$ during the following 40 min, whereas the subjects in the CG were resting. To control for possible circadian rhythm effects, all subjects reported to the laboratory in the morning of the intervention and returned back the same time the day after. Subjects rested between biopsies and stayed in the laboratory until after the 6 h post-exercise biopsy and returned to the laboratory the next morning for the 24 h biopsy. All subjects were given standardized meals the night before, during the day of, and on the morning after the intervention. Muscle biopsy samples were obtained at rest, 30 min after exercise, 2 hrs, 6 hrs and 24 hrs after the end of exercise. In **Paper II**, only samples from two time points, pre and 30 min post, were used.

3.2.5 12-week training study (Paper IV)

Overweight or obese males (40-65 years), who did not exercise regularly and who were interested in participating in the study and eligible to screening ($n=313$), were recruited through newspapers advertisements and advertisements in local occupational health care institutes both from Helsinki and Turku. After a telephone interview, 267 of them were recruited to participate in the screening tests. Their health status and suitability for the study were checked by a health and lifestyle questionnaire, physical examination and an oral

glucose tolerance test. Inclusion criteria were; male sex, age 40-65 years, BMI 25-35 and fasting plasma glucose 5.6-6.9 mmol/l and/or 2-hour plasma glucose 7.8-11.0 mmol/l. Exclusion criteria were earlier detection of IGT and engagement in prescribed diet or exercise programs, engagements in regular and physically very vigorous activities, usage of medication affecting glucose balance (*e.g.* peroral corticosteroid medication). Finally, 144 volunteers were eligible for the study and equally randomized (1:1:1) into one of three groups both in Helsinki and in Turku: (1) a control group (C, $n = 47$), (2) a Nordic walking group (NW, $n = 48$) or (3) a resistance training group (RT, $n = 49$). A total of 115 subjects completed the study (79.9 %) and from those a subgroup of 61 subjects (NW $n=21$, RT $n=22$, C $n=18$) donated muscle samples ($n=55$ in **Paper IV, Preliminary data** include $n=48$). Biopsies were obtained before the intervention period and 48-72 hrs after the last training session after the 12-week intervention period.

During the intervention period, some of the subjects dropped out due to private or medical reasons, difficulties in work arrangements or deterioration of motivation. Participants were advised not to change their habitual diet or their other lifestyle habits during the intervention. If they had been somewhat physically active during their leisure-time, they were asked to continue these habits. The aim of the intervention program was to be additional to normal activity, and should not be taken *e.g.* from other daily life activities. The study subjects were also instructed to avoid alcohol and were advised to keep intake of dietary carbohydrates as normal as possible the 3 days preceding sample collection.

The control group, that had no supervised exercise during the intervention period, was advised, however, about the health benefits of exercise during the first test day.

Both intervention groups trained three times per week for 60 minutes per session during 12 weeks according to special exercise programs in which the exercise intensity was progressively increased after every four weeks of training. All intervention programs were individually designed and supervised.

The resistance training sessions included warm-up exercises by either cycling or rowing with an ergometer for 5 min followed by stretching. In brief, the resistance program focused on strength and power exercises of large skeletal muscle groups, especially those of lower extremities and trunk muscles, but also muscles of upper extremities were trained. Exercise was performed using regular resistance equipment such as machines, dumbbells and barbells. The program included leg press, bench press, leg extension, lateral pull-down, leg flexion and

shoulder flexion, explosive leg squats, squat jumps, standing calf jumps or heel raises. Push-ups, abdominal flexion, and back extension were performed without external weight. External loads started at 50 % of exercise-specific maximal strength (pre-determined by 5RM (RM), repetition maximum) test according to $[(-4.18 \times \text{RM-value of load}) + 103]$ (McDonagh & Davies 1984) and reached 85 % by week nine, which was sustained until the end of the 12th week (week (w) 1-2 at 50 % 2x10 repetitions (reps), w 3-4 at 60 % 3x5 reps, w 5-7 at 70 % 3x5 reps, w 8-9 at 80 % 3x5 reps, w 10-12 at 80-90 % either 3x3, 1x3 reps or 3x5 reps depending on exercise). Training progression was controlled by 5RM strength measurements during the seventh training week. At the end of every session, subjects cooled down by low-intensity cycling or rowing with the ergometer for 5 min and by stretching the main muscle groups.

Before the Nordic walking program began, subjects were familiarized with using the poles in a safe and efficient way. All sessions started with 5 min warm-up exercises (400-500 m walking) and stretching of the main muscle groups. The exercise sessions were carried out at intensity levels increasing from 55 % to 75 % of heart rate reserve (w 1- 4 at 55 %, w 5- 8 at 65 % and w 9- 12 at 75 %). Individual target heart rates were calculated by using measured resting heart rate and the maximal heart rate estimated with the formula $[210 - (0.65 \text{ age in years})]$. Heart rate was monitored during training with Polar F4 (Polar Electro Oy, Kempele, Finland). The target heart rate range was progressively increased. To achieve the desired heart rate target, subjects either increased their walking speed or added uphill walking. After the session, the main muscle groups were stretched during a 5-min cool-down.

3.2.6 Animal model, exercise C57B1/6 mice (Paper II)

To study the BRCA1 expression and regulation in different skeletal muscles, healthy mice were used. Male and female C57Bl/6 mice ranging from 8-10 weeks were utilized in this study. Mice were randomly divided into either an exercise (male n=6; female n=7) group or a sedentary (male n=6; female n=7) group. All animals were treadmill acclimated and then only the exercise group was subjected to an acute bout of treadmill exercise (male= 21.92 ± 0.57 m/min; 40.1 ± 2.75 min; 5 % incline) (female= 26.57 ± 0.30 m/min; 36.5 ± 4.3 min; 5 % incline) while the sedentary animals were placed on the treadmill in a stationary position for an equivalent time. The males were run at a lower speed to maintain similar relative intensities between the males and females.

3.2.7 Animal model, High Fat Diet (Paper II)

To study the influence of high fat diet (HFD) on the BRCA-1 mRNA expression, 12-weeks old C57Bl/6 male mice (n=4/group) were placed on a normal chow diet (10 % kcal fat; D12450K Research Diets) or HFD (45 % kcal fat; D12451 Research Diets) for 10 weeks. At the conclusion of 10 weeks, animals were euthanized and skeletal muscle was harvested, snap frozen in liquid nitrogen, and stored at -80°C.

3.2.8 Human primary myocytes, culturing and stimulation (Paper I, II)

To examine the exercise mimicking effects on skeletal muscle PGC-1 α gene expression, cells were stimulated with either 5-aminoimidazole-4-carboxamide ribofuranoside (AICAR) or beta-adrenergic compounds. In **Paper I**, to start a human primary cell line 50 mg skeletal from the *m. vastus lateralis* was obtained from two female and two male subjects (healthy and normal weight) at rest and stored in sterile phosphate-buffered saline containing 1 % penicillin-streptomycin at 4°C overnight. Extraction of cells from the biopsy sample was performed as described previously (Blau & Webster 1981), with some modifications. In brief, the sample was washed, minced, and dissociated enzymatically in 5 ml of 0.25 % trypsin-1 mM EDTA (all cell media were from Invitrogen, Stockholm, Sweden) at 37°C and 5 % CO₂ with gentle agitation for 20 min. Undigested tissue was allowed to settle for 5 min, and the supernatant was collected in growth medium [Dulbecco's modified Eagle's medium (DMEM-F-12) and 1 % penicillin-streptomycin] containing 20 % fetal calf serum (FCS). Digestion of the slurry was repeated twice. The cells were cultured in T75 flasks (Sarstedt, Stockholm, Sweden), and growth medium was changed every 3rd or 4th day until 60 % confluency was reached. For the experiment, myoblasts were grown in growth medium containing 20 % FCS. At 80 % confluency, the medium was replaced with differentiating medium (DMEM-F-12 and 1 % penicillin-streptomycin) containing 2 % FCS. On day 5 of culture with differentiating medium, the cells were treated with either AICAR (1 mM, Sigma Ald. A1393), norepinephrine (NE; 5 μ M, Sigma Ald. 099K0978), a combination of AICAR and NE, or not treated (control) for 24 h. In **Paper II**, to determine if BRCA1 plays a critical role in the regulation of skeletal muscle metabolic function we reduced BRCA1 expression through shRNA technology. Human skeletal muscle myoblasts were cultured from a *m. vastus lateralis* biopsy from a healthy, lean (normal BMI), 24-year-old female. Low passage number (<7) myoblasts were cultured and upon reaching ~90% confluency, myoblasts were induced to differentiate to myotubes. All cell culture wells were visually examined to ensure

that myotubes covered 90% of the well prior to any experimental utilization. To reduce BRCA1 content in the human myotubes, the cells were transduced with either scrambled shRNA adenovirus (scrambled-shRNA) or adenovirus containing an shRNA sequence targeting the coding region of BRCA1 gene overnight (nt.530-550_NM_007294, shRNA-hBRCA1) and containing a red fluorescent protein (RFP) tag (Vector Biolabs, Philadelphia, PA). After the transduction phase, myotubes were returned to regular growth media for 48 hrs and equivalent adenovirus infection was confirmed via imaging detection of RFP. To confirm reduction of BRCA1 expression, BRCA1 mRNA was isolated from adenovirus-infected human myotubes. Reduction in BRCA1 mRNA expression was confirmed in human myotubes 72 hrs post adenovirus infection using primers for BRCA1 total, BRCA1 Δ 11, and BRCA1 Δ 11b. In **Paper II**, shRNA-BRCA1 or scrambled-shRNA treated human myotubes were also incubated in 30 μ M BSA-conjugated palmitate/oleate mixture to study fat accumulation (See immunohistochemistry section).

3.3 PHYSIOLOGICAL TESTS

3.3.1 VO₂ max

Maximal oxygen uptake was first described by Hill and Lupton back in 1923 (Hill & Lupton 1923) as a measurement of a person's VO₂max and defined the limits of the cardiovascular and respiratory systems ability to transport oxygen. Since then it has been a commonly used test to measure a person's cardiorespiratory fitness. In **Papers I, II and III** an incremental cycle ergometer test until exhaustion was performed to evaluate the subjects' fitness status. During the test, respiratory gases were continuously analyzed (Sensor Medics Vmax 229; Intra Medic AB, Bålsta, Sweden). All subjects rated their effort on a Borg Scale (ranging from 6 to 20) each minute during the test. Subjects in **Papers I and II** with a VO₂max <65 ml·kg⁻¹·min⁻¹ and in **Paper III** with a VO₂max <60 ml·kg⁻¹·min⁻¹ (men) and <50 ml·kg⁻¹·min⁻¹ (women) were included. These inclusion criteria were set since an elite athlete training status might diminish the possibility of detecting significant exercise effects. In **Paper III** the oxygen consumption is presented as VO₂peak instead of VO₂max, since some of the subjects could not for certain reached their VO₂max per definition (Hill & Lupton 1923). VO₂peak is usually easier to define and determine, but its relevance and connection to physiological and pathophysiological status is inferior that of VO₂max.

3.3.2 UKK-test

The UKK Walk Test was originally developed by the Urho Kaleva Kekkonen Institute for Health Promotion Research (the UKK Institute) in Finland. The UKK- test is a 2-km walking test, and a simple and safe physical fitness test designed to measure the respiratory and cardiovascular performance of normally active men and women and is commonly used in patients and subject with higher BMIs. In study IV all included subjects performed the UKK-test on a 200m athletic track. $VO_2\text{max}$ was predicted from the UKK test using gender-specific equations including age, BMI, performance time of the walk and heart rate immediately after the finish (Laukkanen et al. 2000). For $VO_2\text{max}$ values in $\text{ml} \times \text{min}^{-1} \times \text{kg}^{-1}$ the following regression equations were used: $184.9 - 4.65 (\text{time}) - 0.22 (\text{HR}) - 0.26 (\text{age}) - 1.05 (\text{BMI})$ for men, and $116.2 - 2.98 (\text{time}) - 0.11 (\text{HR}) - 0.14 (\text{age}) - 0.39 (\text{BMI})$ for women. Since this test is based on an algorithm, it is not recommended as a test of fitness for extremely fit individuals.

3.3.3 OGTT

The oral glucose tolerance test (OGTT) is a commonly used test to determine the glucose uptake and turnover after an overload of glucose that gives rise to an elevation of glucose levels. In study IV, all subjects were given 75 g of glucose to assess subsequent glucose levels. In brief, after an overnight fast (12 h) blood was taken from the brachial vein before the intervention and after the 12-week intervention period. Immediately after fasting blood samples were taken, and all subjects consumed a bottle of GlukodynR, which consists of 75 g glucose in 330 ml water. Two hours after the participant had consumed the glucose liquid the two-hour glucose sample was taken.

3.3.4 Insulin sensitivity

Plasma glucose was analyzed by enzymatic photometric method (Konelab Glucose HK). Glycated hemoglobin (HbA1c) and serum hs-CRP based on immunoturbidimetry were analyzed with routine standardized methods using a Konelab 20i analyser (ermo Clinical Labsystems Oy, Konelab, Finland). Serum insulin was analyzed by chemiluminescence-immunoassay using an Immulite 1000 analyser (Siemens Medical Solutions, Espoo, Finland). Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as follows:

HOMA-IR fasting serum insulin ($\mu\text{U/mL}$) x fasting plasma glucose (mmol/L) / 22.5 (Matthews et al. 1985).

3.4 BIOCHEMICAL ANALYSIS

3.4.1 Protein extraction

In **all paper** and **Preliminary data** from study 4, human skeletal muscle samples (approximately 20 mg) were homogenized with either glass homogenizers (**Paper I-III**) or by using a bead homogenizer (Retsch MM401) (**Paper IV and Preliminary data**) in homogenization buffer containing: (**Paper I**) 20 mM HEPES (pH 7.5), 0.2 mM EDTA (pH 7.4), 1.5 mM MgCl_2 , 100 mM NaCl, 1 mM Na_3VO_4 , 2 mM dithiothreitol, and 0.4 mM phenylmethylsulfonyl fluoride. NaCl (4 M) was added to a final concentration of 450 mM. After centrifugation at 23,000 g for 10 min, at 4°C, supernatants were mixed with an equal volume of 20 mM HEPES (pH 7.5), 0.2 mM EDTA (pH 7.4), 1.5 mM MgCl_2 , 450 mM NaCl, 1 mM Na_3VO_4 , 2 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, and 40% glycerol, or in RIPA buffer (**Papers II-IV and Preliminary data**) containing: 100 mM NaCl; 50 mM Tris Base; 5 mM EDTA (pH 7.4); 0.5 % Na_3VO_4 ; 0.1 % SDS, 1 % Triton-X100; 1x tablet of complete protease inhibitor cocktail and 1x PhosStop (Roche Diagnostics). The homogenate was gently rotated at 4 °C for 1 hr, followed by centrifugation at 4 °C for 10 min (15,000 g). In the animal study (**Paper II**) mouse skeletal muscle was mechanically homogenized according to previously described techniques (Jackson et al. 2011; Wohlers et al. 2011). In brief, mice skeletal muscle biopsies were homogenized on ice using glass-on-glass homogenizers in RIPA buffer (150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 0.5 % Na_3VO_4 , 0.1 % SDS, 1 % Triton X-100, 1x tablet of complete protease inhibitor cocktail (Roche Diagnostics).

Protein extraction from human differentiating myoblasts or myotubes (**Paper II**), was performed by replacing the medium with 2% horse serum and allowed to incubate until designated time points (myoblasts 48 hrs and myotubes 96 hrs). When cells were harvested, plates were removed, the medium was aspirated, and the cells were washed with ice-cold sterile PBS two times. The cells were lysed and scraped in ice-cold cell lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 10% glycerol, 200 mM NaF, 20 mM sodium pyrophosphate, 10 g/ml leupeptin, 10 g/ml aprotinin, 200 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4). Cell extracts were then rotated for 30

min at 4 °C and centrifuged at 13,000 *g* for 10 min. Total protein was determined in each sample using the Bradford protein assay (human) and the Pierce BCA protein assay (mouse). All samples were stored at -80°C until future analysis.

3.4.2 Immunoblotting

In **Paper I**, skeletal muscle homogenates (40 µg/sample) were separated using 10 % SDS-PAGE. The proteins were blotted onto nitrocellulose membranes (Bio-Rad), and membranes were blocked in 5 % BSA in Tris-buffered saline-Tween 20 (TBST) for 1 h at room temperature and then probed with primary antibodies and diluted in 5 % dry non-fat milk in TBST overnight at 4°C. After being washed in TBST, membranes were incubated for 1 h at room temperature with an anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (New England Biolabs). Bound antibodies were detected using the Immun-Star WesternC Chemiluminescence Kit (Bio-Rad) or the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) according to the respective manufacturer's instructions. Loading uniformity was performed by Ponceau staining. Films were scanned and quantified densitometrically using Quantity One software (Bio-Rad). In **Paper II**, 75 µg of muscle homogenate was loaded on a 6-10 % SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (PVDF). Ponceau staining was used to visualize blots and confirm equal loading of each lane on the gel. Membranes were then blocked with 3 % non-fat dry milk in TBST for 1 h and then probed with antibodies dissolved in a buffer of 5 % bovine serum albumin in TBST on a rocker at 4°C overnight. Following incubation with the primary antibody, membranes were washed in TBST (3 times for 5 min) and then incubated for 1 h with horseradish peroxidase-conjugated rabbit secondary antibody in 3 % or 5 % non-fat dry milk in TBST. Next, membranes were washed in TBST (1 time for 10 min, 3 times for 5 min), followed by chemiluminescence reagent (PierceProtein Research). Membranes were visualized with a chemiluminescence imager (Syngene, Frederick, Md., USA) and quantified with Image J software (National Institutes of Health, Bethesda, Md., USA). In **Paper III**, skeletal muscle homogenates (20 µg protein/sample) were separated electrophoretically on 4-15 % SDS-PAGE gels (BioRad, Stockholm, Sweden) and proteins were then blotted onto PVDF membranes (Millipore, Billerica, MA). The membranes were blocked for 1 h at room temperature in blocking reagent (Millipore) and then incubated with primary antibodies. Beta-tubulin was used as loading control. All primary antibodies were diluted in blocking reagent: ddH₂O (1:1) and incubated overnight at 4°C, and 45 min at room temperature the

day after. After washing with PBS-T (0.1 % Tween 20), membranes were incubated for 1 h at room temperature with IRDye secondary antibody (LI-COR Biosciences, Cambridge, UK). Membranes were scanned using Odyssey SA Infrared Imaging System (LI-COR Biosciences) and quantified using ImageJ.

3.4.3. Enzyme-linked immunosorbent assay (ELISA)

To measure protein levels of the humanin peptide, enzyme-linked immunosorbent assay (ELISA) was used. 100 µg protein of skeletal muscle homogenates were loaded per well, and for serum measurement 100 µl of 1:2 dilution was loaded per well (**Paper IV**, MyBioSource #MBS744343).

To measure protein levels of MOTS-c, RIP140 and MEF2A, skeletal muscle homogenates (between 10-100 µg protein/well depending on the kit) and 100 µl of 1:4 dilution of serum were loaded on a competitive or direct ELISA (**Preliminary data**, MOTS-c; MyBioSource #MBS2033671, RIP140; Blugene #E01N0045/#ABIN1143549, and MEF2A; Wuhan EIAab Science #E9776h/#ABIN1143549). All samples were loaded in duplicates and processed according to the manufacturer's instructions. Plates were scanned using a Microplate Photometer (SYNERGY 2 BioTek, USA, Winooski) with a 450 nm filter.

3.4.3 Immunoprecipitation

To study the interaction of BRCA1 and ACC-p in **Paper II** immunoprecipitation was performed of endogenous BRCA1 protein both in human and mouse tissue. In brief, endogenous BRCA1 protein in mouse (500 µg total protein) or human (150 µg total protein) skeletal muscle homogenate were incubated with 2 µg BRCA1 antibody (I-20, sc-646, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The antigen-antibody complex was combined with protein A affinity and then washed through repeated centrifugation steps. After the final wash the pellet was suspended in sample buffer and heated to 100°C for 5 min. The sample was then cooled and the eluted protein was loaded onto an SDS-PAGE gel for Western blot analysis (see immunoblotting).

3.4.4 Nuclear and Cytoplasmic fractionation

Nuclear and cytoplasmic fractions was performed in **Paper II** and **III** using the Nuclear and Cytoplasmic Extraction Kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Thermo Scientific, 78833). In brief, 10-20 mg of muscle sample was washed twice in PBS and then homogenized in cytoplasmic extraction reagent (CER) I buffer containing protease inhibitors (Complete Mini, Roche Diagnostics). The cytoplasmic fraction was extracted using CER II buffer according to manufacturer's instructions. Pellets containing the nuclear fraction were washed twice in PBS to remove contaminating cytoplasmic proteins before nuclear proteins were extracted in nuclear extraction reagent (NER) buffer. The purity of the fractions was tested using Western blot with antibodies specific for the nuclear protein Lamin A/C and the cytoplasmic protein beta-tubulin.

3.4.5 Immunohistochemistry

To assess whether reduced BRCA1 content leads to accumulation of intra-myocellular lipids shRNA-BRCA1 or scrambled-shRNA treated human myotubes were incubated in 30 μ M BSA-conjugated palmitate/oleate mixture in DMEM for 4 hrs as previously described (Timmers et al. 2012). Myotubes were then stained with BODIPY (Molecular Probes, Carlsbad Calif, USA) and imaged at 20 x magnification using a Zeiss Axiovision 4 (Zeiss, Oberkochen, Germany) as previously described (Spangenburg et al. 2011).

3.4.6 RNA extraction

Total RNA was extracted from skeletal muscle biopsies and from cells using the Trizol® reagent, based on the acid phenol method earlier described (Chomczynski & Sacchi 1987). The integrity of total RNA was determined using 1% agarose gel electrophoresis. Total RNA was analyzed (concentration and quality) spectrophotometrically by measuring absorbance at 260 nm (Nano- Drop 2000; Thermo Scientific, Gothenburg, Sweden). In brief, each sample was homogenized with a polytron on ice (**Papers I, II and III**) or by using a bead homogenizer (Mixer mill Retsch MM400, Haan, Germany) (**Paper IV and Preliminary data** from study 4). Total RNA was precipitated using isopropanol, the final RNA pellet diluted in RNase-free water and stored at -80°C.

3.4.7 Primer design and isoform detection

Table 2. Primer sequences for designed primers used in this thesis.

Amplicon Name	Forward Primer Sequence	Reverse Primer Sequence
GAPDH	ACAGTTGCCATGTAGACC	TTTTTGGTTGAGCACAGG
Total PGC-1 α	GTGGTGCACTGACCAATGAG	CTGCTAGCAAGTTGCCTCA
PGC-1 α -ex1a	TGTATGGAGTGACATCGAGTGT	GCTGGTCTTCACCAACCAGA
PGC-1 α -ex1b	GACACACATGTTGGGGTTATCA	ACCAACCAGAGCAGCACATTT
trunc-PGC-1 α	CCACACACAGTCGCAGTCACA	GTCACCTGGAAGATATGGACAT
non-trunc-PGC-1 α	CCACACACAGTCGCAGTCACA	GGGAACCCCTGGGGTCATTTG
Brca1 mouse	CACAG GTATGCCAGAGAAA	ATCCTGGGA GTT TGCATTTG
BRCA1 total human	TAGGGCTGGAAGCACAGAGT	AATTCCTCCCAATGTTCC
BRCA1 Δ 11 human	GATTTGAACACCACTGAGAAGCGT	CAGAGGAGTCACTTATGATGGAAGGG
BRCA1 Δ 11b human	AACCACAGTCGGGAAACAAGCAT	TTCTGACCAACCACAGGAAAGCC
Total LIPIN-1	ACATGAACACATCTGAGGATGAG	TAGGTGTTGAAGGTCGGGAAC
LIPIN-1 α	ACATGAACACATCTGAGGATGAG	AGGTCGGGAACCGGAAGGACTG
LIPIN-1 β	ACATGAACACATCTGAGGATGAG	TCCGAAGGATGGAACAGGGA
NCoR1-1	TGACAACCTCTTACAGCAGCA	GGGCTTGACAGCTTCAACTTC
NCoR1-2	ACTACTAAAGGATGCCAGTAACATT	ATGTCTTCCAGCACAAAGATGA
NCoR1-3	GGATGTGTCCAAAACAAAAGAGATA	TGGTGATTCTGCTGTGGTC
MT-RNR2	AATCACTTGTTCTTAAATAGGGACC	GAACCCCTCGTGAGCCATT
MT-RNR2L1	CACTTGTTCTTAAATAGGGACTTGTC	AGCTGAACCCCTCGTGAGGC

In **Paper I**, we identified PGC-1 α splice variants transcribed from the canonical and the proposed upstream-located promoter (exon 1a and 1b). This was done by using the NCBI genome database and modified PGC-1 α primers to fit the human genome from mouse primers published previously (Chinsomboon et al. 2009). In **Paper III**, we used primers designed by Ydfors *et al.* 2013 (Ydfors et al. 2013) to measure the exercise response in two additional PGC-1 α splices (trunc-PGC-1 α and non-trunc-PGC-1 α). For a detailed description of the different PGC-1 α splice transcripts, see Ydfors *et al.* 2013 and Fig. 7 below.

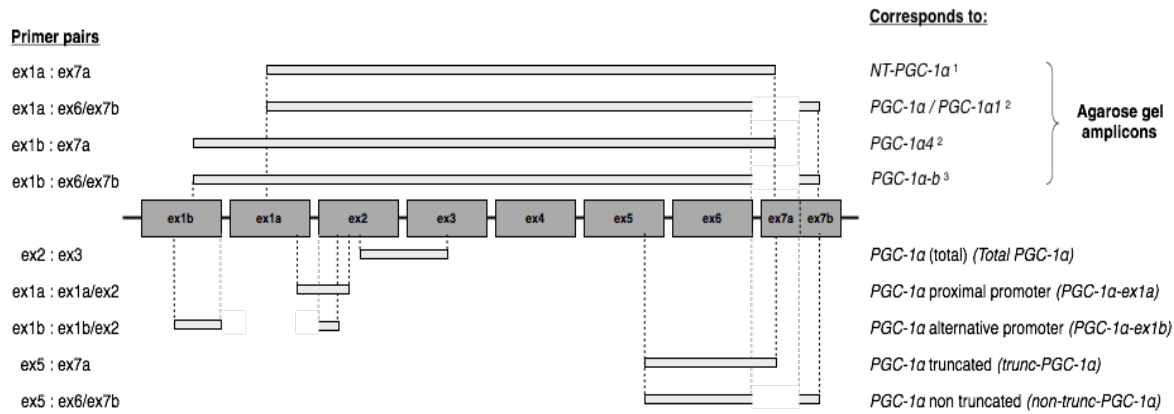


Figure 7. Schematic representation kindly provided by Ydfors *et al.*, 2013. The figure displays exons 1–7 of the human PGC-1 α gene. Primer pairs are depicted to the left, and the resulting splice variants measured and promoter sites are depicted to the right. Upper panel: resulting amplicons with RT-PCR. Lower panel: amplicons measured with real-time RT-PCR. Exon 1b is transcribed from the alternative promoter, and exon 1a is transcribed from the proximal promoter. Exon 7a (ex7a) is the exon insert resulting in the truncated forms of PGC-1 α (trunc-PGC-1 α) and exon 7b (ex7b) is present in non-truncated PGC-1 α (non-trunc-PGC-1 α). The corresponding names of previously described splice variants are stated to the right in the upper panel and some of them are used in Paper I. ¹Zhang *et al.* (2009), ²Ruas *et al.* (2012), ³Miura *et al.* (2008).

In **Paper III**, we also identified and measured LIPIN-1, LIPIN-1 α , and LIPIN-1 β , based on the first publication that described LPIN1 splice variants in the mouse (Péterfy *et al.* 2005). All primers for detection of human LPIN1 transcripts were designed by alignment of mouse and human sequences from the NCBI genome database similar to that of the PGC-1 α splices in **Paper I**, see Fig. 8. By using the NCBI genome database we also found isoforms of NCoR1 that have not previously been analyzed in skeletal muscle, primers were design for NCoR1-1, NCoR1-2, and NCoR1-3 isoforms respectively, see Fig. 8. In **Paper II**, the two documented variants of the BRCA1 gene, BRCA1 Δ 11 or BRCA1 Δ 11b, were studied by designing primers as previously described (Wilson *et al.* 1997), for sequences see Tab. 2. All primer used in this thesis were designed to cover exon-exon boundaries to minimize amplification of genomic DNA.

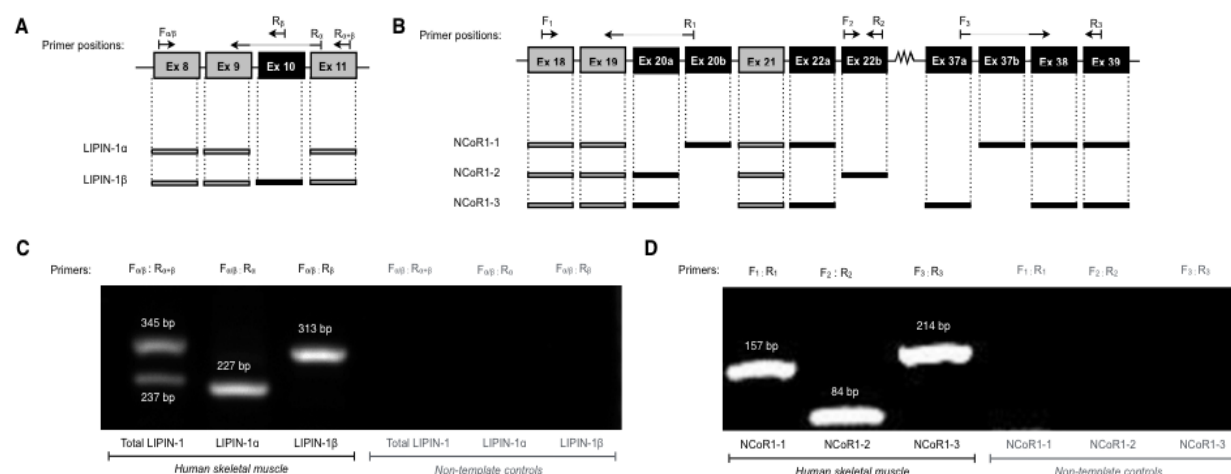


Figure 8. Description of LIPIN-1 and NCoR1 primers (A-B), and visualization of RT-PCR products (C-D) shown. For detailed description see **Paper III**.

3.4.8 Reverse transcription polymerase chain reaction (RT-PCR)

RNA was reverse transcribed (total volume of 20 µl) using random hexamer primers (Roche Diagnostics, Mannheim, Germany) and 2 µg of total RNA and the Superscript reverse transcriptase kit (Life Technologies, Stockholm, Sweden (**Papers I, II and III**)) or 1 µg of total RNA and the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA (**Paper IV and Preliminary data** from study 4)). Samples were stored at -80°C until further use.

3.4.9 Quantitative real-time PCR (qRT-PCR)

qRT-PCR was used for mRNA quantification in **all Papers** included in the thesis and in accordance with the MIQE guidelines (Bustin et al. 2009). For gene transcripts shown in Table 1, SYBR green analysis was performed (SYBR Green PCR Master Mix, Applied Biosystems). The total reaction volume was 10-15 µl, containing 2-5 µl cDNA sample. Final concentration of the primers was 0.3-0.4 µM (for details see specific Papers) and all samples were loaded in duplicates. All quantification reactions were controlled with a melting curve, and primer efficiency was tested using standard curves. For quantification of the remaining factors, TaqMan Gene Expression Assays (Applied Biosystems) were used. Total reaction volume for TaqMan analysis was 10-25 µl, containing: 2-5 µl cDNA sample; 5-12.5 µl TaqMan Fast Universal PCR Master Mix (Applied Biosystems); and 0.5 µl gene-specific

primers. All samples were loaded in duplicates. Reaction volume varied dependent on the plate and machine used (96-well MicroAmp Optical plates, Applied Biosystems 7500 Fast Real-Time PCR System or the 384 well Hard-Shell PCR plate with the Bio Rad CFX384 Real Time System C100 Touch Thermal Cycler, for details see specific Paper of interest). In **Paper I**, 18S rRNA was selected as an endogenous control, in **Papers II** and **IV** glyceraldehyde dehydrogenase (GAPDH) was used. In **Papers III** and **IV** 40S ribosomal protein S18 (RPS18) was used (not published) as an additional endogenous control to GAPDH. The cDNA concentration, annealing temperature and thermocycling conditions were optimized for each primer pair and gene target, and assay sensitivity was high for all PCR products ($RSq > 0.99$, and efficiency $> 90\%$) (Schmittgen et al. 2000). For each subject, all samples were simultaneously analyzed in the same assay. The expression of each target gene was then evaluated by the threshold cycle (C_t), also called the quantification cycle (C_q), method ($2^{-\Delta C_t}$). This method provides the level of expression of the target gene relative to the level of expression of the reference gene in each sample (Schmittgen et al. 2000; Bustin et al. 2009).

3.4.10 DNA extraction

In **Paper IV** DNA was isolated with a modification of the Gentra Puregene Tissue Kit (Qiagen). In brief, 5-10 mg skeletal muscle tissue was homogenized in lysis buffer (Puregene cat.# D-5002) with proteinase K (Qiagen cat.# 19131), and incubated at 55°C for 1 h. Protein was precipitated followed by centrifugation 3 min at 14,000 g. DNA was subsequently precipitated using ice-cold isopropanol. DNA was washed in 70 % EtOH and mixed with 50 μ l of DNA hydration solution (Puregene cat.# D-5004). After incubation at 65°C for 1 h, the concentration and quality of the DNA was analyzed using a Nanodrop® 2000 spectrophotometer (NanoDrop 2000; Thermo Scientific, Gothenburg, Sweden).

3.4.11 mtDNA and nuclear DNA fraction

The ratio of mtDNA to nuclear DNA (nucDNA) have been used as a marker for mitochondrial content (Sparks et al. 2005; Swerdlow et al. 2006; Rabøl et al. 2009; Guo et al. 2009; Tong et al. 2011). The mitochondrial DNA content per genome was then calculated as the ratio of the mtDNA to the genomic DNA for each sample.

Total DNA was isolated as described above. Analysis were performed in 384-well Hard-Shell PCR plates (#HSP3951, Bio Rad), with sample duplicates, using the Bio Rad CFX384 Real Time System, C100 Touch Thermal Cycler. DNA was diluted to a concentration of 10 ng/ul and for mtDNA analysis samples were diluted 1:10000. The total reaction volume was 10 µl, containing: 2 µl DNA sample; primer forward (final concentration 0.3 µM); primer reverse (final concentration 0.3 µM); and SYBR Green PCR Master Mix (Applied Biosystems). Cycle parameters: one cycle of 95°C for 3 min, followed by 40 cycles at 95°C for 10 s and at 60°C for 30s, followed by one cycle 95°C for 10 s, 65°C for 5 s and lastly 95°C for 5 s. In **Paper IV**, human nuclear DNA was analyzed by measuring the Myogenin promotor (forward primer: AGGTGCTGT CAGGAAGCAAGGA, reverse primer: TAGGGGGAGGAGGGAACAAGGA) and mitochondrial DNA was analyzed measuring mitochondrially encoded cytochrome c oxidase I gene (COX1, forward primer: CCCCTGCCATAACCCAATACCA, reverse primer: CCAGCAGCTAGGACTGGGAGAG) (Rabøl et al. 2009).

3.4.12 Enzymatic activity analyses

3.4.12.1 *Citrate synthase*

Citrate synthase (CS) is a rate-limiting metabolic enzyme of the TCA that has been shown to reflect the mitochondrial content of skeletal muscle (Holloszy et al., 1970). A well-established adaptation of skeletal muscle to endurance training is an increase in mitochondrial density (Holloszy et al. 1970; Holloszy & Booth 1976; Duscha et al. 2012), which enables muscle to produce more aerobic energy. In this sense, CS activity can be used to obtain an objective, biochemical indication of the training response. CS activity was measured in **Paper IV**. The assay was performed according to the fluorometric principles of Lowry & Passonneau (1972) (L. Lin et al. 1988). In brief, a section of a biopsy was homogenized in 0.1 M phosphate buffer (pH 7.7) with 0.5 % BSA. For CS analysis, wet tissue lysates were added to a reagent solution (0.1 M Tris-HCl, 2.5 mM EDTA, 0.5 mM L-malate, 512.5 nM NAD⁺, 399 µg MDH). 50 µg acetyl-CoA started the reaction and the velocity was registered with a fluorometer (reduction of NAD⁺ to NADH). A standard curve computed from known amounts of NADH was subsequently used to determine the CS activity. Correction for wet muscle weight was performed (Vigelsø et al. 2014; Sahin et al. 2006).

3.4.13 Mitochondrial respiration and ROS measurements

3.4.13.1 *Agilent Seahorse*

In **Paper II**, mitochondrial oxygen consumption rates were measured in shRNA-BRCA1 or scrambled-shRNA treated human myotubes similar to a previously described technique (Schuh et al. 2012; Jackson et al. 2013). Bioenergetic analyses of isolated human myotubes were performed using an XF24-3 Extracellular Flux Analyzer (Seahorse Bioscience). All cells were cultured and transduced in Seahorse 24-well XF Cell Culture Microplates.

After calibration of the XF24-3 Extracellular Flux Analyzer, the microplate containing the myotubes transduced with scrambled shRNA or shRNA specific to human BRCA1 was placed in the analyzer. Basal oxygen consumption rate (OCR; pmol/min) were initially quantified across both conditions in assay measurement buffer (MB) at 37°C contained 120 mM NaCl, 3.5 mM KCl, 1.3 mM CaCl₂, 0.4 mM KH₂PO₄, 1 mM MgCl₂, 5 mM HEPES (pH 7.4) supplemented with 2.5 mM D-glucose (Sigma G7528) and 0.5 mM L-carnitine (Sigma CO158). Mitochondrial respiration was induced with either albumin (03117405001; Roche, Indianapolis, IN) conjugated sodium palmitate (palmitate 100 µM, P9767; 50 M; Sigma) or sodium pyruvate (10 mM, P8574; Sigma). A second identical treatment of substrate was initiated after 20 min and OCR was again recorded. Following the last OCR measure induced by the second exposure of substrate, 400 nM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, C2920; Sigma) a known inhibitor of mitochondrial complex III, was injected to assess non-mitochondrial OCR measures. OCR measures presented are the average values detected after the OCR reaches a steady state following the introduction of the substrate or FCCP. Basal OCR values presented are taken immediately prior to the first injection of either pyruvate or palmitate. The mean derived for each group was determined by collecting the average OCR values from 7-10 different wells. This results in 25-30 independent measures per group as we previously described.

3.4.13.2 *ROS measurements in shRNA-BRCA1 myotubes*

ROS are generated during mitochondrial oxidative metabolism as well as in cellular response to xenobiotics, cytokines, and bacterial invasion. Oxidative stress refers to the imbalance due to excess ROS or oxidants over the capability of the cell to mount an effective antioxidant response. Increased oxidative stress as evidenced by increased 2',7'-dichlorofluorescein (DCF) signal can be used to detect increase ROS production/accumulation. In **Paper II**,

Scrambled-shRNA or shRNA-BRCA1 treated human myotubes were placed in DCF supplemented KRB buffer for 30 min at 37°C and then washed 3 times. To measure ROS the global ROS indicator H2-DCF was used. H2-DCF signal was quantified using a fluorescent plate reader (H2, Biotek, Burlington, VT). The myotubes were also visually imaged using an inverted epifluorescence microscope (Zeiss, Oberkochen, Germany).

3.4.14 Insulin signaling and glucose uptake in shRNA-BRCA1 myotubes

In brief, Ad-shRNA-BRCA1-RFP or AD-shRNA-RFP treated human myotubes were (in **Paper II**) serum starved for 4-5 hrs as previously described (Wohlers et al. 2013). Myotubes were then washed three times and incubated with or without 50 nM insulin in warm Krebs Ringer (135 mM NaCl, 10 mM NaHCO₃, 5 mM KCl, 3 mM CaCl₂, 2 mM MgSO₄, 1.2 mM NaH₂PO₄) excluding glucose for ~30min at 37°C. At the conclusion of the insulin incubation myotubes were exposed to 50 μ M 2-NBDG with or without 50 nM insulin for ~30 min at room temperature as previously described (Wohlers et al. 2013). Myotubes were then washed three times and placed in room temperature Krebs Ringer and 2-NBDG fluorescence (438 nm excitation; 535nm emission) and RFP fluorescence (556 nm excitation; 586 nm emission) measures were recorded using a BioTek Synergy plate reader (BioTek, Winooski, VT). All 2-NBDG measures were normalized to RFP values.

To examine the insulin signaling in human myotubes, Scrambled-shRNA or shRNA-BRCA1 myotubes were serum starved for 4 hrs in DMEM. Myotubes were then either control treated or treated with 50 nM insulin for 30 min. Protein was then isolated (as previously described (Spangenburg 2005)) from control treated or insulin treated scrambled-shRNA or shRNA-BRCA1 human myotubes.

3.5 STATISTICAL ANALYSES

In **all Papers** in this thesis, differences were considered statistically significant at $P < 0.05$. Data are presented as means \pm SEM and as means \pm SD or means (range) when presenting the included study subject characteristics.

In the acute exercise study with or without restricted blood flow (**Paper I**) a two-way analysis of variance (ANOVA) was used for the repeated mRNA measurements (pre, 0 h and 2 hrs

post) in the two exercise conditions (R-leg and NR-leg). In the cell culture experiments a one-way ANOVA was used to identify gene expression changes. Planned comparison was used to reveal any internal differences even if there was no overall significance. Since there were only a few samples in the protein analysis, a nonparametric Friedman's analysis of variance using ranks for multiple dependent samples was used to evaluate the effects of time (pre, 0 h and 2 hrs post) in the exercise condition (R-leg and NR-leg) respectively. In the acute exercise study and the animal models in **Paper II**, repeated measurements ANOVA was used and employed with Tukey's HSD post-hoc tests. Paired and unpaired t-tests were also used.

Linear mixed-effects models (LMM) enable the modeling of correlated data without desecration of important regression assumptions and is efficient in repeated measurements analysis and when data are unbalanced (n differ between the intervention groups). This type of statistical analysis was used in **Papers III and IV**. In the acute exercise study with repeated biopsies over 24 hrs (**Paper III**), LMM was used both for gene expression and protein analysis with factors time (pre and at 30 min, 2 hrs, 6 hrs and 24 hrs post) x group (exercise and control), followed by pairwise comparison within groups using Fisher's least significant difference (LSD) test. The nuclear and cytoplasmic protein analysis was only performed in the exercise group and thereby analyzed using one-way ANOVA to identify changes from baseline.

In the 12-week training study in men with impaired glucose regulation (**Paper IV**), both gene expression and total protein abundance were analyzed using LMM with factors time (pre and post) x group (exercise and control). If interactions were significant, the analyses were followed by pairwise comparison within groups using LSD test and to correct for multiple testing, Šidák correction was performed. In **Paper IV**, changes between groups regarding fitness and metabolic parameters were analyzed with one-way ANOVA. CS activity measurements were only applicable in the resistance and the control group and changes in CS activity were tested with student's t-test. Associations between fitness and metabolic variables were assessed by Pearson's correlation test at baseline and compared to both humanin protein levels at baseline and Δ humanin protein. In **Papers III and IV**, outliers were defined using the Inter-Quartile range (IQR) as observations $<Q1-(1.5 \times IQR)$ or $>Q3+(1.5 \times IQR)$ and excluded from the analysis.

3.6 METHODOLOGICAL AND STATISTICAL CONSIDERATIONS

3.6.1 Repeated biopsies

The skeletal muscle is a perfect tissue to study from a research perspective since it can grow, adapt and able to donate small pieces without any seriously harm. In this sense, skeletal muscle tissue is really impressive and a genial formed tissue to do research on. Skeletal muscle biopsies are used for diagnosis in patients undergoing evaluation for inflammatory, metabolic, or mitochondrial myopathies (Goutman & Prayson 2013) . However, in this thesis all biopsies were taken on healthy young individuals in all the studies except for study 4 in which pre-diabetic older men were included. Performing exercise interventions and studying the molecular response in skeletal muscle usually requires repeated biopsy samplings. Therefore, it is important to bear in mind that the sampling by itself can elicit some molecular changes affecting the results obtained. Repeated biopsies over hours, days or even weeks and years are a common feature in the exercise physiology field. Also, how the muscle tissue is affected by the invasive biopsy procedure has over the years been explored. In 1992 Staron *et al.* showed that a visual focal damage was induced by the biopsy procedure and that this damage was not completely repaired after 2 weeks (Staron et al. 1992).

Another potential confounding factor in performing exercise studies in humans is the fact that multiple muscle biopsies in human skeletal muscle can induce immunological responses 24-48 hrs after the initial biopsy (Malm et al. 2000). It is highly likely that the procedure associated with the biopsy *per se* evokes a stress response within the tissue. However, a biopsy taken within 24 hrs after a first biopsy seems less likely to be affected since the inflammation response might not yet have had any influence. A study by Lundby *et al.* 2005, showed that the mRNA responses of specific genes were not affected when two to three biopsies had been obtained previously from the same muscle over a 5-6 h period as typically used in gene expression studies (Lundby et al. 2005). They also showed that a similar mRNA response was observed in the muscle from which 2 or 3 biopsies had already been obtained and in the muscle where no biopsy had previously been taken. This was evident both for genes directly involved in fat and carbohydrate metabolism and carbohydrate oxidation. Also, the mRNA of factors coupled to angiogenesis regulation and stress responsive genes was shown to be unaffected by the prior biopsies in the same muscle (Lundby et al. 2005). However, more recent findings highlight that that mRNA changes in the non-exercising skeletal muscle tissue seems mostly to involve factors linked to inflammation, myogenesis,

mitochondrial biogenesis, and glucose metabolism, findings which are in contrast to the study by Lundby *et al.* (Lundby et al. 2005; Friedmann-Bette et al. 2012; Van Thienen et al. 2014). However, Lundby *et al.* did not use a control group, rather they used an intra individual control. No matter the study design, the specific gene studied should always be compared to base line, to a control group or to a housekeeping gene. A recent study by Boman *et al.* concluded that the fiber type composition differed somewhat between biopsies taken from the same leg but there were no within-subject differences in gene expression between the 3 different biopsies where the base line biopsy were obtained 1 h prior to the other biopsies (Boman et al. 2015). To conclude, first; in contrast to mRNA analysis, protein measurement are more stable and seem not to be as affected by the biopsy procedure itself and secondly; gene expression analysis after repeated biopsies separated by a few hours seems less affected by the previous sampling than after 24 hrs due to delayed inflammation response.

3.6.2 Protein analysis

When measuring protein abundance with a semi-quantitative method (Western blot) the specificity of the primary and second antibodies is of high importance. In **Paper III** we had huge problem with some of the commercial available antibodies for both LIPIN1 and NCoR1 proteins. We tried both published and unpublished available antibodies for both of these proteins but could not detect bands with the certainty that we wanted (band in the right size span on the membranes and low amount of unspecific binding) and could therefore not use these data. This resulted in limitations since we only could study the gene expression instead of the actual protein and its time-course after an acute bout of exercise. In this study, we also performed immunoprecipitation but antibodies that had high binding affinity for the protein quantifications (and other antibodies of different brands etc.) did not work for the immunoprecipitation protocol (which was modified and also different protocols were tested). Therefore, we could not continue with these analyses which limited the possibility to see actual interactions of different proteins and transcriptions factors. In **Papers II** and **III** precast gels were used for the immunoblotting, which can be regarded as more safe and more reproducible than preparing own gels as was done in **Paper I**. Factors that always should be Also to bear in mind when using Western blot for protein quantifications is the following; possible non-specific protein bindings, the insignificant chance of the target protein to react with the secondary antibody and posttranslational phosphorylation or oxidation, all of which could result in multiple bands or bands on a different size. Other issues that can affect the

analysis include bad gels, dehydrated sandwich and not optimized transfer procedure (time- and temperature-wise) which might lead to proteins not transferred to the membrane or just partly transferred. Problems with skewed bands can also appear if bubbles are present during the transferring of sample from the gel to the membrane and then incubated with antibodies. The amount of methanol in the transfer buffer have also been shown to affect the transfer of proteins.

In this sense, it is of high importance to always modify and optimize the protocol for each specific protein of interest which has carefully been done during all included Western blot analysis in this thesis.

In **Paper IV** and **Preliminary results** from study 4, direct or indirect ELISA was used instead of Western blot for the protein analysis. ELISA can be recommended when measuring protein abundance in a large number of samples as in study IV. ELISA analysis is dependent on high specificity of an antibody, in a combination with a fluorescent enzyme that can be converted to a signal and detected. The specificity in the ELISA kit used in this thesis was very high, and measurements of total protein concentrations were performed both on serum and on skeletal muscle homogenate. ELISA does not display any molecular weight of the analyzed protein but instead measures the amount (concentration) of the bound protein to a specific antibody by an absorbance technique. However, one advantage of Western blotting is that it is less likely to give false positive results, which might be a risk using ELISA since you do not see the molecular weight of the captured protein.

3.6.3 To choose a statistical method

Choosing the right statistical method is sometimes a hazard when conducting research. In all of the Papers in this thesis, statistical approaches and analysis have been discussed at length. However, in the light of this thesis I would like to state some changes that I might have done if the analysis were performed again today. In brief, we performed logarithmic transformation to the base 10 of the gene expression data to ensure normal distribution (**Paper I**). This analysis method compresses data a lot and I think using the natural logarithm (\ln) would have been more accurate. Since data of the protein analysis was performed using a nonparametric test (Friedman's analysis of variance) data should have been shown as median instead of mean values. Though we wanted to identify changes in mRNA expression and did not really know what to expect we should have used pairwise comparison instead of planned

comparison. And lastly, in **Paper I** we also write “planned comparison was used (i.e., post hoc test)” which is not accurate since planned comparison is not a post hoc test per definition. Also, even though Friedman’s test is generally considered to be a rank randomization equivalent of repeated measures ANOVA it is more a sign test (Zimmerman & Zumbo 1993). Therefore, a Man-Whitney U test might have been better than Friedman’s test since it was two different conditions and repeated biopsy time points. A combination of both Kruskal-Wallis and Friedman’s test might also have been use otherwise, since there is no applicable test in this sense for nonparametric data. In **Paper I**, subject characteristics were shown as means (range) and it would have been more appropriate to show median (range).

4 RESULTS AND DISCUSSION

In four different experimental settings, this thesis has explored factors proposed to be of importance for exercise-induced mitochondrial biogenesis and fat oxidation in human and mouse skeletal muscle. This thesis aims to explore the interconnected network of transcription factors and co-regulators that control the expression of genes encoding mitochondrial proteins, including those controlling replication and transcription of the mitochondrial genome. The main focus is on the expression of the transcriptional co-activator PGC-1 α and its surrounding network. Three specific hypotheses of this thesis were that;

- different PGC-1 α transcripts have a markedly differential response to exercise
- BRCA1 regulate fatty acid oxidation
- mitochondrial-derived peptides are affected by exercise

4.1 EXERCISE-INDUCED EXPRESSION OF PGC-1 α

4.1.1 Ischemic exercise

PGC-1 α has been shown to be important for the regulation of mitochondrial biogenesis and angiogenesis in response to regular exercise, with increased oxidative capacity and oxygen delivery as a consequence (D. A. Hood 2006; Arany et al. 2008; Chinsomboon et al. 2009). In **Paper I**, the acute response in skeletal muscle after an ischemic exercise condition was investigated. This was done by applying an increased external pressure which results in a reduction in oxygen saturation as described in the method section 3.2.3. It was, for the first time in human skeletal muscle, shown that PGC-1 α can be transcribed from different promoters (the canonical and an alternative upstream-located promoter) resulting in splice variants of the PGC-1 α transcript (PGC-1 α -ex1a and PGC-1 α -ex1b, in **Paper I** called PGC-1 α -a and PGC-1 α -b), see Fig. 7 for nomenclature and descriptions.

Interestingly, the PGC-1 α -ex1b transcript was almost undetectable at baseline, but had a highly-expressed PCR product after exercise (**Paper I**, Fig. 2). This indicates that PGC-1 α -ex1b could be involved in the initial adaptational process that occurs in skeletal muscle in response to exercise, but may not have regulatory functions at rest.

In **Paper I**, it was also demonstrated that different PGC-1 α transcripts responded differently to an ischemic stimulus. A single bout of knee extension exercise with restricted blood flow

(R-leg) led to a seven-fold increase of the PGC-1 α -ex1a mRNA and over a 100-fold increase 2 hrs post exercise of the PGC-1 α -ex1b mRNA ($p=0.05$; Fig. 3 A and B in **Paper I**). This was not only the first time it was shown that the different PGC-1 α transcripts are transcribed from different promoters but also that different training stimuli affected these transcripts in different ways. It was also demonstrated that the pre-exercise expression of the PGC-1 α -ex1b splice variant was very low compared to that of the PGC-1 α -ex1a and that training with non-restricted blood flow (NR-leg) did not elicit any expressional changes of either splice variant (Fig. 2, **Paper I**). The finding that the total PGC-1 α is more affected by ischemic training was first described and published by our group in 2004 (Norrbom et al. 2004). The results from **Paper I** provide further support for this previous finding. Recently, studies have shown that short burst of sprint-like training and high-intensity interval training (HIIT) can activate PGC-1 α in a similar way as endurance type training (Burgomaster et al. 2008; Gibala & McGee 2008; Little et al. 2010; Hoshino et al. 2016). Reduced blood flow leads to an enhanced metabolic stress, why the ischemic training performed in study 1 should be considered as high, relative intensity, even though the absolute workload was quite low.

In support of the notion that exercise with restricted blood flow induced a marked metabolic perturbation is that it has been shown to increase skeletal muscle levels of lactate and ATP metabolites and a 2 to 3-fold increase in the levels of circulating catecholamines (Sundberg & Kaijser 1992; Sundberg 1994). This increased metabolic profile and an increased O₂ extraction seen with restricted blood flow in our previous studies (Sundberg & Kaijser 1992), have also been shown in athletes performing isokinetic maximal contractions (Paradis-Deschênes et al. 2016). In the study by Paradis-Deschênes *et al.* the training performed was classified as high-intensity resistance exercise and a much higher external pressure (200 mmHg) was employed (Paradis-Deschênes et al. 2016). Blood flow restriction in a resistance model in mice has also been shown to increase the expression of known targets for exercise-induced transcription such as AMPK, a PGC-1 α activator (Xu et al. 2016). This supports our finding that the activation of PGC-1 α might reflect the amount of metabolic stress induced and not only the type of exercise *per se*.

However, this might be a simplistic explanation and a recently published study, comparing blood flow restricted cycling to regular cycling and resistance training, could not detect any metabolically beneficial effects when exercise was performed with restricted blood flow (Smiles et al. 2017). However, the exercise performed was relatively short, 15 min compared to our 45 min of exercise, and the external pressure used was much higher than what we applied (90 mmHg). In that study, they did not measure the training response of PGC-1 α , but

studied factors coupled to autophagy *i.e.* intracellular processes important for preserving cellular homeostasis by degrading nutrient substrates, protein aggregates etc. in response to “stress” conditions, such as strenuous contractile activity. It should be noted, however, that the same research group in a previous study measured PGC-1 α and its splice variants with the same exercise protocol and did not find any difference between restricted blood flow training compared to regular endurance training (Conceição et al. 2016). If this lack of PGC-1 α response was due to the exercise duration or the exercise type (cycling compared to our one-legged exercise) remains unknown, but the differences between those studies and **Paper I** might imply that a longer endurance-based occlusion stimulus may be required to modulate MAPK signal transduction (p38 MAPK kinase phosphorylation increases in our study) and downstream gene transcription of *e.g.* PGC-1 α . In conclusion, PGC-1 α is affected by ischemic exercise and it appears that intensity *i.e.* metabolic stress, might be a crucial factor for determining the magnitude of the PGC-1 α response. Also, in response to exercise, PGC-1 α -ex1b seems like the most exercise-responsive transcript with hitherto unknown importance.

4.1.2 Aerobic and resistance exercise

In this thesis, three different exercise settings were applied to study changes in PGC-1 α transcript and protein levels with both acute exercise and prolonged training. Interestingly, as stated above, non-restricted blood flow exercise did not induce any significant changes in any of the three PGC-1 α transcripts examined in **Paper I**, 2h post exercise compared to Pre. This could probably be explained by the low absolute exercise intensity performed in the non-restricted blood flow condition. In contrast to this, subjects in **Paper II** performed an acute and quite intense 60-min exercise bout on a cycle ergometer. In **Paper II**, the mRNA expression of Total PGC-1 α , PGC-1 α -ex1a, PGC-1 α -ex1b, trunc-PGC-1 α and non-trunc-PGC-1 α were significantly elevated at 30 min, 2hrs and 6 hrs after exercise. With the exception of PGC-1 α -ex1a, all transcripts differed significantly from the control group ($p < 0.01$; **Paper II**, Fig. 2 A, B, D, and E).

The magnitude of the exercise response of the PGC-1 α -ex1a transcript was different compared to the other transcripts. This indicates that the PGC-1 α -ex1a transcript is not as sensitive to an exercise stimulus as the other transcripts. This notion is supported by the fact that we and others have previously shown similar exercised induced gene expression

differences of these transcripts (Norrbom et al. 2011; Ydfors et al. 2013; Lundberg, Fernandez-Gonzalo, Norrbom, et al. 2014; Silvennoinen et al. 2015). In **Paper III**, the protein expression of PGC-1 α and PGC-1 α -ex1b were examined. This was the first time that the PGC-1 α -ex1b protein was shown in humans. Interestingly, the temporal pattern differed between the two proteins. Even though there was no significant interaction between the exercise and the control group the PGC-1 α -ex1b protein responded and peaked much earlier than that of PGC-1 α (**Paper III**, Fig. 7A and B). Total PGC-1 α protein levels were significantly increased at 24 h after the exercise bout (1.2-fold, $p < 0.05$; **Paper III**, Fig. 7A) and PGC-1 α -ex1b protein levels were increased 3.1-fold at 30 min compared with Pre ($p = 0.05$; **Paper III**, Fig. 7B). The early peak seen for the PGC-1 α -ex1b protein might reflect a rapid mRNA translation into protein or an increased posttranslational stabilization, resulting in a positive protein turnover. Further, this finding could lead to the speculation that the activation, rather than increased expression of PGC-1 α , mediates the initial phase of the exercise-induced increase in mitochondrial biogenesis. An early onset and increased levels of PGC-1 α protein is in accordance with previous findings in animal and human skeletal muscle (Wright, Han, et al. 2007; Perry et al. 2010).

PGC-1 α protein levels after both acute exercise and prolonged training has been studied before (Perry et al. 2010; Little et al. 2011), but without including a control group. In this regard, it is important to remember that several different stimuli can trigger the expression of PGC-1 α , *i.e.* the induction is not solely dependent on exercise (Puigserver et al. 1998; Chinsomboon et al. 2009; Norrbom et al. 2011; Ruas et al. 2012) as seen as an induction of PGC-1 α mRNA also in the control group in **Paper III**. However, study 3 could not observe any significant nuclear accumulation of PGC-1 α or PGC-1 α -ex1b protein after acute exercise, which is in contrast to earlier findings (Little et al. 2011; Safdar et al. 2011). In a recent study looking at circadian effects of genes and proteins coupled to mitochondrial oxidation, no change over a 24 h time-course with five repeated biopsies was seen of PGC-1 α protein (van Moorsel et al. 2016), which is in line with our results in the control group.

In **Paper III** it was concluded, in accordance with **Paper I**, that PGC-1 α mRNA (**Paper I and III**) and protein (**Paper III**) levels are rapidly elevated by exercise and emphasizes PGC-1 α -ex1b as the most exercise-responsive PGC-1 α isoform. The mRNA levels all of the different PGC-1 α transcripts were back to baseline at the 24 h time point in **Paper III**. Therefore, in study 4 we chose to only look at total PGC-1 α and PGC-1 α -ex1b expression since these are the most exercise sensitive ones. Even though the exercise intensity

progressed during the 12 weeks of the study, no significant change in total PGC-1 α or PGC-1 α -ex1b expression were seen after the intervention compared to before, or between the aerobic, resistance or the control group (**Preliminary data** from study 4, Fig. 9). The lack of PGC-1 α response in this study might be explained by the post exercise biopsy time point (24-72 hrs after the last training session) demonstrating restoration to baseline levels of PGC-1 α . Importantly, the magnitude of increase after each training session has been shown to be progressively decreased, resulting in a stair-case type response over repeated training sessions (Perry et al. 2010). Therefore, changes in basal levels of PGC-1 α after prolonged exercise training interventions are more relevant to study at the protein level.

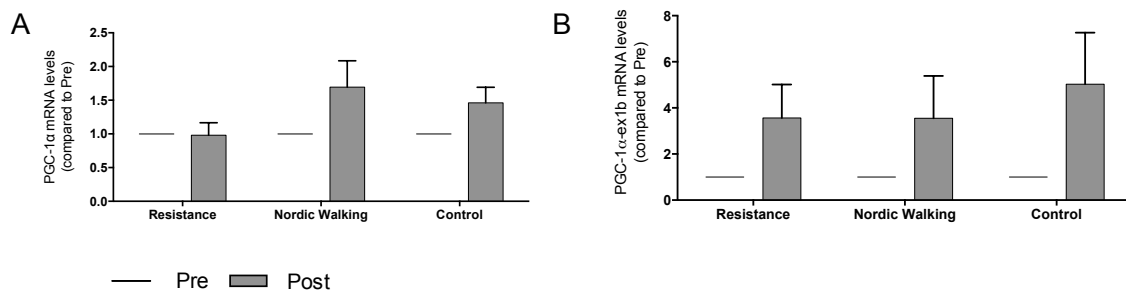


Figure 9. Preliminary data, fold change levels of PGC-1 α (A) and PGC-1 α -ex1b (B) mRNA before and after 12-weeks of training, study 4. (RT n= 17, NW n=12 and CON n= 16). Values are presented as means \pm SEM.

4.2 REGULATORY NETWORK OF PGC-1 α

This thesis mainly focuses on PGC-1 α , and by studying PGC-1 α *per se* as well as its upstream regulators and downstream targets, and their response to different types and dosage (duration and intensity) of exercise training. The purpose was to broaden the perspective on the surrounding network of this “master-switch”. Several signaling pathways have been suggested to regulate the PGC-1 family. These signaling pathways includes calcium-regulated pathways, calcineurin and CaMK, and p38 AMPK as well as activation of β 2-adrenergic receptors (Winder & Hardie 1996; Zong et al. 2002; H. Wu et al. 2002; Handschin et al. 2003; Kusuhara et al. 2007; Wright, Geiger, et al. 2007; Miura et al. 2007; Chinsomboon et al. 2009; Tadaishi, Miura, Kai, Kawasaki, et al. 2011).

4.2.1 Upstream signaling, activators and coactivators affecting PGC-1 α

PGC-1 α expression levels are regulated by a plethora of stimuli, such as the mechanical stretching of the muscle fiber, changes in calcium flux and increased metabolic demand reflected by an elevated AMP/ATP ratio. The results from **Paper I**, clearly demonstrates that factors coupled to the calcium-activated pathway increased significantly in expression and phosphorylation (activity) in response to the intervention (**Paper I**, Fig. 4 A-C). Interestingly, there were no differences in the induction of MCIP1 mRNA (marker for calcineurin activation) and p38 mitogen-activated protein kinase (MAPK) protein phosphorylation 2 hrs after exercise when comparing the restricted and non-restricted blood flow conditions. This might argue against calcineurin and p38 MAPK as candidates for the sole regulation of PGC-1 α in active skeletal muscle. This argument is also supported by other studies challenging the importance of Ca²⁺ signaling for the activation of PGC-1 α in the exercising muscle (Garcia-Roves et al. 2006; Ojuka et al. 2003; Vaarmann et al. 2008).

Studies in humans and animals have shown a link between Ca²⁺ signaling, basal levels of Ca²⁺ and the activation of PGC-1 α , as well as a connection to increased mitochondria density (Chin et al. 1998; Olson & Williams 2000; H. Wu et al. 2002; Bruton et al. 2010; Place et al. 2015). Interestingly, in contrast to MCIP1 and p38 phosphorylation, AMPK phosphorylation increased significantly only in the condition with restricted blood flow *i.e.* higher relative exercise intensity (**Paper I**, Fig. 4 C). This finding suggest that the AMPK pathway might be a strong candidate for the regulation of exercise-induced transcription of PGC-1 α . To investigate this further, cultured human satellite cells were treated with AICAR (an AMPK analog) and/or norepinephrine (NE, a strong β -adrenergic stimulator). AICAR stimulation increased the mRNA expression of both the PGC-1 α -ex1a and PGC-1 α -ex1b splice variants as well as the Total PGC-1 α (**Paper I**, Fig. 5). A significant increase in the mRNA levels of PGC-1 α -ex1a, PGC-1 α -ex1b and Total PGC-1 α were also seen with the combination of AICAR and NE, and with NE treatment alone. Noteworthy, the PGC-1 α -ex1b mRNA expression increased most in the cells treated with a combination of AICAR and NE. Acute β -adrenergic stimulation has been shown to be inefficient in altering PGC-1 α expression in resting human skeletal muscle (Tadaishi, Miura, Kai, Kawasaki, et al. 2011).

It should be pointed out that the resting biopsy in the leg subsequently exercising in a non-restricted fashion was obtained at the same time point as the biopsy obtained 2 hrs post

exercise in the leg trained with restricted blood flow (study 1). This means that the tissues (Pre in the NR-leg and 2 hrs post in the R-leg) had been exposed to the same levels of circulating catecholamines but there was no induction of PGC-1 α -ex1b in the NR-leg at the Pre time point (**Paper I**, Fig. 3). Since almost all tissues throughout the body are exposed to exercise-induced changes in the circulating levels of catecholamines, it is unlikely that β -adrenergic stimulation alone can regulate muscle-specific remodeling in any major way. More likely, skeletal muscle adaptation, *e.g.* mitochondrial biogenesis and angiogenesis, is highly specific to the skeletal muscle conducting the exercise and not solely dependent on hormonal fluctuations. This supports a strong role of AMPK in the exercise-induced upregulation of PGC-1 α , and especially the PGC-1 α -ex1b transcript. AMPK has been shown to phosphorylate PGC-1 α and initiate its activity (Jäger et al. 2007), but interestingly it seems like the activation of AMPK on the different PGC-1 α promoters might differ (Popov et al. 2017). Using administration of metformin as an AMPK activator, in an acute exercise trial in men it was recently shown that low-intensity exercise markedly increased the expression of PGC-1 α mRNA via the alternative promoter (PGC-1 α -ex1b), without increasing ACC^{Ser79/222} (a marker of AMPK activation) and AMPK^{Thr172} phosphorylation (Popov et al. 2017). However, since that study used low-intensity exercise, we cannot exclude the possibility that substantial activation of AMPK might occur after high-intensity exercise or ischemic stress, that subsequently affects the expression of PGC-1 α mRNA via the alternative promoter (PGC-1 α -ex1b).

Further, studies in rodents have shown that β -adrenergic receptor agonists and antagonists have strong effects on the expression of PGC-1 α mRNA via the alternative promoter, probably dependent on the cAMP responsive element-binding protein-1 (CREB1) signaling pathway (Chinsomboon et al. 2009; Tadaishi, Miura, Kai, Kano, et al. 2011; Wen et al. 2014). In support of the hypothesis that β -adrenergic stimulation may not act on its own, a study performed in rats treated with the β -adrenergic agonist clenbuterol for 3 weeks showed a decrease in PGC-1 α mRNA and protein levels, in mitochondrial enzyme activity and in markers for mitochondrial content compared to a control group (Hoshino et al. 2011). However, this long-term administration, mimicking long-term stress, might also downregulate stress-induced factors such as PGC-1 α as a regulatory feedback mechanism. The true effect of β -adrenergic stimulation on PGC-1 α is still fairly unknown, confusing and perplex.

Other studies have also reported increased levels of PGC-1 α mRNA with compounds such as clenbuterol (Miura et al. 2008; Gonçalves et al. 2009). This tells us that the regulation of PGC-1 α is very diverse and factors that can be activated by PGC-1 α can also in some ways regulate PGC-1 α itself.

In this sense, a factor that both act on and is regulated by PGC-1 α is the tumor protein p53. It has been shown that p53 can directly bind to the PGC-1 α promoter and thereby increase PGC-1 α expression (Aquilano et al. 2013). Also, p53 is activated/phosphorylated by muscle contraction and metabolic stress leading to nuclear and mitochondrial translocation of p53 and resulting in the transcription of metabolic genes (Saleem et al. 2013; Saleem & D. A. Hood 2013). Metabolic stress that results in p53 activation and triggers cell-cycle arrest, ROS clearance or apoptosis can also be regulated by PGC-1 α binding to p53 (Sen et al. 2011). This binding of PGC-1 α to p53 modulates the transactivational function, resulting in preferential transactivation of pro-arrest and metabolic target genes of p53. This allow the regulation of p53 function by the PGC-1 α protein to be seen as a critical switch in determining the p53-mediated cell fate. PGC-1 α is in that sense defining the p53 response to metabolic stress.

In **Paper III**, there was a significant interaction between the exercise group and the control group as well as over time for p53 mRNA. Interestingly, p53 mRNA was significantly induced at some of the time points in the control group, which might indicate a biopsy effect (metabolic and/or inflammatory stress of the tissue) (**Paper III**, Fig. 4 C). Nevertheless, 6 hrs after exercise the induction of the p53 transcript peaked (2.9-fold, $p < 0.01$) and this time point was not significantly elevated in the control group. The acute response of the p53 transcript and protein is still not fully elucidated after this study, *e.g.* protein was not measured which is necessary for the understanding of the activation and temporal resolution of p53 after one exercise bout. Acute exercise studies in animals have revealed an important function of p53 in the metabolic response to training and as an important player in the mitochondrial adaptation to exercise (Saleem & D. A. Hood 2013; Saleem et al. 2013). Understanding the full potential of p53 in response to exercise and in a metabolic perspective in human muscle is still warranted.

Neither PGC-1 α (Total PGC-1 α), nor its isoforms can bind directly to the DNA. Instead, they interact with DNA-binding transcription factors to exert their function. Numerous factors have been suggested to co-regulate and co-activate PGC-1 α . One of these factors is the lipid metabolism enzyme LIPIN-1. In **Paper III**, we could was shown for the first time

that two splice variants of the LIPIN-1 transcript (LIPIN-1 α and LIPIN-1 β) exist in human skeletal muscle (see Fig. 8) similar to what previously had been reported in mouse adipocytes (P  terfy et al. 2005).

LIPIN-1 interacts with and binds to PGC-1 α and co-regulates its transcriptional function (Reue & P. Zhang 2008; Kim et al. 2013) and plays a role in skeletal muscle mitochondrial adaptation after exercise in rodents (Higashida et al. 2008). In **Paper III**, LIPIN-1 gene expression and the exercise adaptive response were studied. Acute exercise significantly elevated skeletal muscle LIPIN-1 and LIPIN-1 α , but not the LIPIN-1 β mRNA expression (**Paper III**, Fig. 3 A-B), compared to the control group. The expression pattern over time was rather similar for all the LIPIN-1 transcripts in the exercise group. Similar to p53, an induction of the LIPIN-1 mRNAs was detected in the control group. The significant difference in expression in the control group at the 24 h time point indicated that it was not an artifact due to diurnal changes, but rather something due to the sampling process *e.g.* inflammation. Studies in human skeletal muscle myotubes and in myoblasts have implied a link between LIPIN-1 and inflammation, which might be a possible explanation (Michot et al. 2013; Meana et al. 2014).

Repeated biopsies have been performed previously in both animal and human studies. In many exercise studies with repeated biopsies, the possible diurnal changes or effects of inflammation are not accounted for. Multiple muscle biopsies in human skeletal muscle have been shown to induce immunological responses at 24 hrs or 48 hrs after the initial biopsy (Malm et al. 2000), which might explain the induction at the 24 h time point of some of the factors in **Paper III**. We included a non-exercising control group in an attempt to isolate the exercise-specific effects. The inclusion of this control group thereby minimized the impact of *e.g.* biological variability, biopsy effects, and diurnal changes that previously published studies have highlighted (Staron et al. 1992; Malm et al. 2000; Lundby et al. 2005; Lamia et al. 2009; Martin et al. 2010; Van Thienen et al. 2014; van Moorsel et al. 2016). However, the exercise response of p53 and LIPIN-1 are still not fully understood. It may be concluded from **Paper III** and the study by Friedmann-Bette *et al* 2012 that, if an intra-individual control or a separate control group is not used, false positive results might be found when using repeated biopsies to conclude mRNA expression changes in exercise interventions (Friedmann-Bette et al. 2012).

The majority of the mRNAs that changed in the non-exercising skeletal muscle tissue appears to involve factors linked to inflammation and glucose metabolism but could certainly involve

more factors and pathways. However, a recent study looking at gene expression and fiber type variations in repeated biopsies from the *m. vastus lateralis*, concluded that gene expression differs significantly between individuals but is not affected by repeated muscle biopsy sampling from the same subject (Boman et al. 2015). Thus, from a methodological perspective it is of value to study gene expression over time after repeated biopsies within a subject as was done in this thesis as long as a control group is included.

Another way that the PGC-1 α activity can be regulated and affected is by transcriptional modulation and initiation or by binding to a PGC-1 α functional protein. Coactivators such as PPAR γ , NRF-1, and NRF-2 are key targets of PGC-1 α mediated coactivation, however myocyte enhancer factor-2 (MEF2) and the estrogen-related receptors (ERRs) have also been shown to be important coactivators for PGC-1 α (Finck & Kelly 2006). In **Paper III**, no exercise mediated effect on NRF-1 or ERR α mRNA (**Paper III**, Fig. 4 A and D) could be observed. This lack of NRF-1 induction is consistent with a previously performed acute exercise study which also included repeated biopsy sampling over a time course of 24 hrs (Pilegaard et al. 2003). Together, these data together indicate that NRF-1 might not be acutely regulated by exercise.

Mice constantly overexpressing NRF-1 have been shown to present increased levels of MEF2A and the glucose transporter type 4 (GLUT4) (Baar et al. 2003) which indicate a potential role for NRF-1 in the metabolic regulation and exercise adaptation. In **Paper III**, an exercise-mediated effect of the ERR γ gene expression could be detected, with a peak at the 6-hr time point (**Paper III**, Fig. 4 B). This might reflect that ERR γ is more important than ERR α as an acute exercise-response target gene. ERR γ has been linked to AMPK and is presumably important in promoting exercise-induced metabolic adaptations (Rangwala et al. 2010; Narkar et al. 2011). The activity of the ERRs, especially ERR α , has been shown to be inhibited through interaction with the RIP140 protein, a corepressor that antagonizes the action of PGC-1 α (Qi & Ding 2012). This suggest that the role of the ERRs in the oxidative adaptation to exercise might be of greater importance than initially thought.

In study 4, protein and mRNA analysis of MEF2A was performed before and after 12 weeks of training. It is known that the PGC-1 α promoter has binding sites for MEF2A (Czubryt et al. 2003; Baldán et al. 2004) and can induce the transcription of the PGC-1 α gene. **Preliminary data** from study 4, indicates that 12 weeks of resistance training significantly increased the protein levels of MEF2A (see Fig. 10), but no correlation of Δ MEF2A protein and Δ PGC-1 α or Δ PGC-1 α -ex1b mRNA could be detected. MEF2A mRNA levels were not

significantly changed in any of the intervention groups in study 4 which is not surprising since the post biopsy was taken 24-72 hrs after the last training session. This lack of MEF2A mRNA change has also been shown in a previous study, in which no changes were detected 0 hr or

3 hrs after an acute bout of exercise (Barrès et al. 2012). This indicates that the window for detecting the MEF2A gene activation might have been missed, and that an expression change could be speculated to occur between 3 hrs and 24 hrs after an exercise bout.

A weak correlation was detected between Δ MEF2A and Δ HOMA-IR ($r=-0.454$, $p=0.007$) in men with IGR (**Preliminary data**, study 4). Even though the correlation was weak, it is strengthened by earlier exercise studies, both in humans and in animals, showing that MEF2A is coupled to GLUT4 expression, and that this interaction is coupled to increased binding of MEF2A to the promoter of the GLUT4 gene (Smith et al. 2007; Gong et al. 2011; E. A. Richter & Hargreaves 2013) and therefore important for glucose regulation.

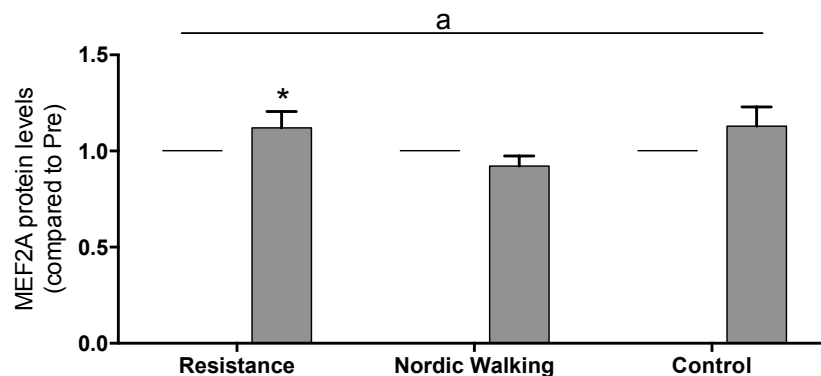


Figure 10. Preliminary data, fold change MEF2A protein levels before and after 12-weeks of training, study 4. ^a $p \leq 0.05$ with factors time \times group (LMM). * $p \leq 0.05$, compared to Pre (pairwise comparison (Sidak correction) within groups). (RT $n=14$, NW $n=14$ and CON $n=14$). Values are presented as means \pm SEM.

To conclude, upstream signaling mechanisms are important to study further in the attempt to understand the different functions of PGC-1 α and how it is activated. Different types of training regimes and stimuli appear to determine which upstream pathway that is activated. Therefore, immunoprecipitation experiments are needed to understand the protein-protein interactions that might affect PGC-1 α activation.

4.2.2 PGC-1 α inhibitors and corepressors

Corepressors are proteins that mediate the repressive activity of nuclear receptors and other transcription factors. RIP140, is a nuclear receptor interacting protein and a corepressor involved in a spectrum of metabolic pathways such as glucose uptake, glycolysis, fatty acid oxidation, Krebs cycle, and oxidative phosphorylation (Seth et al. 2007; Frier et al. 2011). Previous studies have demonstrated that RIP140 and PGC-1 α have opposite effects such that transcription factors activated by PGC-1 α are repressed by RIP140 (Hallberg et al. 2008; Chen et al. 2012). Also, a direct protein-protein interaction between PGC-1 α and RIP140 has been observed (Gupta et al. 2008; Hallberg et al. 2008).

In **Paper III**, the effect of acute exercise on this corepressor of PGC-1 α was studied. There was no significant difference between the exercise and the control group regarding RIP140 gene expression ($p=0.06$), even though the expression was significantly increased at all time points, compared to baseline levels, in the exercise group. This mRNA induction of RIP140 with exercise is in line with previously published human studies (Frier et al. 2011; Edgett et al. 2013). RIP140 protein levels differed significantly between the exercise and the control group, and interestingly RIP140 protein was significantly elevated in the control group at 2 hrs, 6 hrs and 24 hrs after the first biopsy (**Paper III**, Fig. 6 A). Furthermore, a previous acute HIIT study looking at RIP140 protein at 0 hrs and 3 hrs post exercise did not find any changes in RIP140 protein in human skeletal muscle (Frier et al. 2011). Taken together, this might indicate that exercise halts the inhibitory effects of RIP140 on the oxidative machinery and that the biopsy-induced stress *per se* might activate RIP140. In **Paper III**, cellular location analysis of RIP140 protein was performed and it was found that RIP140 was solely located within the nucleus, leading to speculation that this might strengthening its corepressor ability and activity (**Paper III**, Fig. 7 H).

In study 4, **Preliminary data** of RIP140 protein showed no significant differences over time nor any interactions between the intervention groups, but RIP140 mRNA data was significantly decreased in the resistance group over time, see Fig. 11. However, RIP140 protein levels decreased in all subjects except one in the control group and a trend towards increased levels in the resistance group could be seen ($p=0.06$, Fig. 11). Noteworthy, it has been shown in human and rat skeletal muscle that a RIP140 decrease is not required to get an increase in mitochondrial content, and 2 weeks of HIIT training did not change RIP140 protein levels in human skeletal muscle (Frier et al. 2011). Altogether, this supports our

finding that exercise does not reduce RIP140 protein content but that exercise *per se* might reduce the stress-induced effect from the biopsy as was found in **Paper III**.

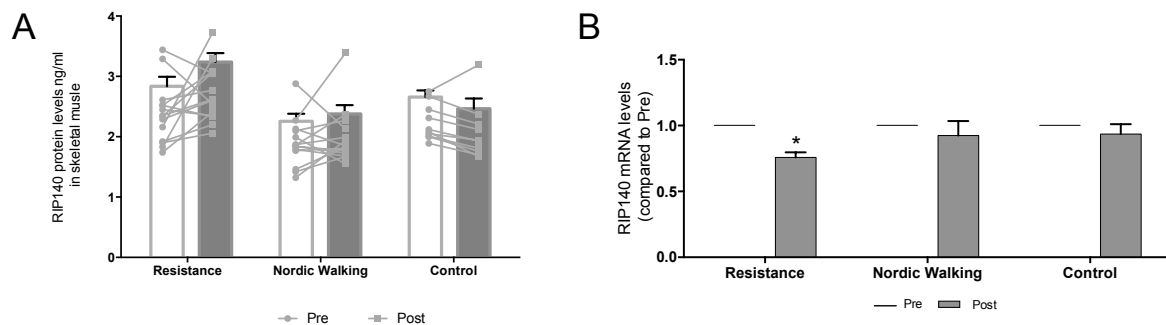


Figure 11. Preliminary data, RIP140 protein levels in human skeletal muscle (RT n= 14, NW n=14 and CON n= 10) (A), and fold change RIP140 mRNA levels (RT n= 17, NW n=12 and CON n= 16) (B) before and after 12-weeks of training, study 4. ^ap ≤ 0.05 with factors time x group (LMM). *p ≤ 0.05, compared to Pre (pairwise comparison (Sidak correction) within groups). The bars represent means ± SEM.

Another transcriptional corepressor shown to interact with proteins involved in the oxidative and metabolic machinery is nuclear receptor corepressor 1 (NCoR1). In **Paper III**, it was shown for the first that there are three different isoforms of NCoR1 (NCoR1-1, NCoR1-2 and NCoR1-3) expressed in human skeletal muscle, see Fig. 8.

Similar to RIP140, the program orchestrated by NCoR1 has been postulated to antagonize that of PGC-1α (Yamamoto et al. 2011; Perez-Schindler et al. 2012). To what degree NCoR1 (or its splice variants) might exert inhibitory actions on PGC-1α or on other exercise-specific factors after acute exercise in humans could not be elucidated from study 3.

However, studies in animals have shown that genes involved in mitochondrial biogenesis and oxidative phosphorylation are up-regulated in a muscle-specific knock-out model of NCoR1 and that NCoR1 seems to specifically antagonize the actions of PGC-1α, ERRs and MEF2 (Perez-Schindler et al. 2012). These common pathways make NCoR1 an interesting target for the understanding of the metabolic adaptation in skeletal muscle after exercise. NCoR1 has been shown to regulate histone acetyltransferases (HATs) and histone deacetylases (HDACs) (You et al. 2013; Mottis et al. 2013). HATs have been shown to usually stimulate gene transcription, whereas HDACs typically repress gene expression. Since both NCoR1 and HDACs have been linked to regulation of the muscle adaptation machinery and the fact that NCoR1 KO-mice show increased muscle volume, muscle mass and strength as well as an

increased mitochondrial capacity (Yamamoto et al. 2011; Mottis et al. 2013; Muoio 2017) it is of profound importance to study NCoR1 further in human exercise studies.

4.2.3 Downstream targets of PGC-1 α

In connection to PGC-1 α , nuclear-encoded mitochondrial proteins are located furthestmost downstream in the proposed machinery of exercise-induced mitochondrial biogenesis. In **Paper III**, some of these factors were measured in an attempt to study if they were activated with acute exercise and if the expression pattern correlated with PGC-1 α mRNA or protein.

In **Paper III**, there was a clear induction of VEGF-A mRNA in the exercise group (**Paper III**, Fig. 5 A) at all time points except at 24 hrs when the levels were back to baseline. VEGF-A has been shown to peak between 3-6 hrs post exercise in previously publications from our research group and others (Breen et al. 1996; T. Gustafsson et al. 1999; T. Gustafsson et al. 2005). Somewhat surprising, the VEGF-B expression did not respond in the same way as VEGF-A and no exercise-mediated effect could be seen (**Paper III**, Fig. 5 B). It should be noted that an absence of exercise-induced VEGF-B expression has previously been observed, but at 30 min and 48 hrs after an exercise bout (Kivelä et al. 2007). Following acute exercise the TFAM mRNA expression was upregulated while TFB1M and the marker for mitochondrial oxidative capacity SDH subunit-A (SDHA) were unaffected (**Paper III**). In an earlier study from our lab it has been demonstrated that the basal levels of the TFAM protein differed when comparing elite athletes and moderately active individuals (Norrbom et al. 2010), indicating its involvement in the regulation of oxidative capacity. The same study from Norrbom *et al*, showed a marked increase in the TFB1M mRNA after 10 days of training, indicating that the gene expression regulation of TFB1M might be regulated by repeated endurance training and, as shown in study 3, and not acutely regulated after one single exercise bout.

4.3 METABOLIC FUNCTION AND REGULATION IN SKELETAL MUSCLE

The metabolic requirements of skeletal muscle are highly variable and capable of adapting to increasing or decreasing energy demands as well as to mechanical loading and unloading.

Skeletal muscle metabolism is also under hormonal control, *e.g* by insulin and sex hormones such as estrogen (Spangenburg et al. 2012).

In **Paper II**, the role of the BRCA1 gene and protein expression in skeletal muscle metabolism after exercise was explored using a translational approach. In addition to fat metabolism, skeletal muscle is also a major glucose-regulating tissue. In study 4, a longitudinal exercise intervention was performed to examine the effects of markers coupled to glucose regulation and metabolism in skeletal muscle from persons with IGR (**Paper IV** and **Preliminary data**).

4.3.1 BRCA1, existence and action in skeletal muscle

4.3.1.1 Sex and skeletal muscle differences in BRCA1 expression

The BRCA1 gene produces either a full-length BRCA1 or two variants through alternative splicing, BRCA1 Δ 11 or BRCA1 Δ 11b (Wilson et al. 1997). In **Paper II**, both BRCA1 isoforms were identified and present in both human and mice skeletal muscle (**Paper II**, Fig. 1 mice data and Fig. 3 human data). Neither the BRCA1 mRNA levels (full-length and splice variants) nor the BRCA1 protein level differed in expression between sedentary women and men (**Paper II**, Fig. 3 A-C). The short BRCA1 Δ 11 protein was exclusively detected in the cytosolic fraction of the myocyte. This is in line with previously published data showing that the short isoforms lacks a nuclear localization signaling element (Wilson et al. 1997; Mok & Henderson 2010).

In mice, there seems to be both a sex and muscle type specific difference in the BRCA1 protein expression. The *m. gastrocnemius* in male mice had much greater levels of both the full-length and the short (BRCA1 Δ 11, 85 kDa) BRCA1 splice variant compared to females (**Paper II**, Fig. 1D). These findings were somewhat surprising since BRCA1 is a well-established estrogen-sensitive gene (Clemons & Goss 2001; Wang & Di 2014). But it might be speculated that the higher BRCA1 content in male mice might be due to the lower concentrations of estrogen with a compensatory mechanism to regulate fat oxidation.

Full-length BRCA1 protein levels did not differ between *m. gastrocnemius* and *m. tibialis anterior* (TA), but was undetectable in the *m. soleus* (**Paper II**, Fig. 1 E and F). This lack of BRCA1 in the *m. soleus* was very unexpected in this highly oxidative muscle. Male mice had significantly higher levels of the short BRCA1 Δ 11 in the TA and *m. soleus* compared to the *m. gastrocnemius* but in female mice no differences were detected in BRCA1 Δ 11 protein

content among the different muscles. In this regard, the high levels of the short BRCA1 Δ 11 in the *m. soleus* might be suggested to compensate for the absence of the full-length BRCA1 protein.

Estrogens have a powerful influence on both fat and carbohydrate utilization and metabolism in peripheral tissue, thereby significantly affecting metabolic flexibility in women (Campbell & Febbraio 2001). This flexibility effect seems to result in an ability to maintaining both a greater insulin sensitivity as well as having a greater capacity to oxidize lipids (Høeg et al. 2011). As a consequence, younger women have been shown to exhibit a greater difference in the respiratory quotient (RQ) from a fasted to a fed state compared to men (Høeg et al. 2011), and interestingly this effect seems to be opposite in older women and men with a different estrogen profile (Toth et al. 1998). This is in accordance with several studies showing that women use more of the oxidative system coupled to fat oxidation than men both at rest and during exercise (Horton et al. 1998; Friedlander et al. 1998; Carter et al. 2001; Maher et al. 2009; Lindholm et al. 2014).

However, metabolic flexibility in women is impaired in response to loss of circulating estrogens during menopause, thus increasing the risk for the development of type 2 diabetes and the metabolic syndrome (Campbell & Febbraio 2001). What role BRCA1 plays in the metabolic differences seen between the sexes still necessitates further investigations.

4.3.1.2 BRCA1 regulation of fat oxidation

Analysis in **Paper II** clearly showed that both acute exercise and high fat diet could regulate and elicit changes in BRCA1 protein and mRNA expression. An acute bout of exercise resulted in a significant increase in ACC-p/ACC total compared to before exercise in human skeletal muscle (**Paper II**, Fig. 4 A and B). There was considerable inter-individual variability in the magnitude of interaction between ACC-p and BRCA1 in both women and men at baseline and after exercise but no significant difference between the sexes could be detected (**Paper II**, Fig. 4 C). This lack of difference in the interaction between ACC-p and BRCA1 might be due to the small sample number. By pooling the data from both women and men, an increased protein interaction of ACC-p and BRCA1 could be detected as an exercise-response (**Paper II**, Fig. 4 D-E). In mice subjected to an acute bout of treadmill exercise, a significant elevation of the ratio of ACC-p/ACC-total and an increased BRCA1-ACC-p protein-protein interaction in both female and male *m. gastrocnemius* were found, compared to sedentary mice. Previously, studies have assigned MaCoA as a potent allosteric inhibitor of

carnitine palmitoyltransferase 1 (CPT-1) and that it serves as a precursor for lipid synthesis (McGarry et al. 1983; Winder & Hardie 1996). As anticipated, MaCoA content significantly decreased in response to the exercise bout in the female animals, but surprisingly, the increase in ACC-p levels did not correspond to a decline in MaCoA levels in the male mice (**Paper II**, Fig. 2 C). However, MaCoA levels in sedentary female skeletal muscle were significantly higher compared to sedentary males. In human breast cancer cells the interaction of BRCA1 and ACC-p has previously been described as an important step in the regulation of lipid metabolism (Magnard et al. 2002; Moreau et al. 2006). Taken together, this emphasizes that exercise might inactivate ACC in both humans and mice and thereby increase the fatty acid transport over the mitochondrial membranes. To confirm this, re-analysis of data from a previous study (Thomson et al. 2007) which showed that AICAR stimulates a decrease in MaCoA content, was performed. This re-analysis showed that female mice had higher levels of resting MaCoA and had a greater response to AICAR treatment than age-matched male mice. The exercise effect on MaCoA needs further evaluation but it is possible that exercise might decrease the levels of MaCoA at least in female mice. Whether there is a similar sex difference, at rest or after exercise, in humans could unfortunately not be concluded from these results. Even though MaCoA could not be measured due to limited biopsy material in study 2, acute exercise has previously been shown to reduce the MaCoA content in human muscle after exercise (Dean et al. 2000; Frøsig et al. 2009).

Still, the regulatory mechanisms of BRCA1 might be more divergent in its action and it may not influence the fatty acid transportation, it might rather act as part of a broader signaling network. The effect of the exercise-mimicking compound AICAR in human myotubes was studied by silencing BRCA1 expression using shRNA (**Paper II**). It was clearly demonstrated that AICAR induced ACC phosphorylation when BRCA1 levels were reduced (**Paper II**, Fig. 7 A and B). However, lower BRCA1 content resulted in a significant increase (~20 %) in basal phosphorylation of AMPK and also increased basal phosphorylation of ACC by ~70 %. This inactivation of ACC has previously been shown to be mediated by AMPK (Winder & Hardie 1996). Taken together, this suggests that BRCA1 might not be required but rather act as an additional player in ACC phosphorylation.

Data from **Paper II**, also demonstrates that loss of BRCA1 protein (using shRNA) resulted in accumulation of intramyocellular lipids and reduced insulin-induced glucose uptake (**Paper II**, Fig. 5 B and D) in human myotubes. These cells also exhibited reduced Akt (protein kinase B) phosphorylation in response to insulin treatment compared to the insulin response in the control myotubes, which strengthens the notion that BRCA1 is involved in glucose

regulation. Apart from the glucose regulation, human myotubes with reduced BRCA1 expression exhibited lower basal oxygen consumption rates (OCR) compared to scrambled-shRNA myotubes (**Paper II**, Fig. 6 A and B). When human myotubes were treated with conjugated-free fatty acids, an additional increase in lipid storage in myotubes with reduced BRCA1 expression compared to control was observed. Also, myotubes transduced with shRNA-BRCA1 had higher reactive oxygen species (ROS) levels, indicating a high intracellular stress.

To summarize, these data suggest that reduced BRCA1 content in human myotubes results in decreased insulin signaling, increased lipid storage, reduced mitochondrial function, and enhanced ROS production. In conclusion, the observations from **Paper II** provide new evidence for a potential role of BRCA1 as a metabolic regulator in skeletal muscle and as an important player in fatty acid metabolism.

4.3.2 Changes in metabolic regulation following exercise in persons with pre-diabetes

In **Paper IV**, fitness parameters and metabolic markers such as peak VO_2 , fasting insulin, HOMA-IR and HbA_{1c} were improved significantly in the aerobic training group (Nordic Walking) after the intervention (**Paper IV**, Table. 1). Due to limited material citrate synthase activity (CS, a marker for mitochondrial capacity), could not be measured in the aerobic training group, but a trend towards increased mtDNA:nucDNA ratio (29 %) was observed. In the resistance group, CS activity increased significantly and there was also a trend towards an increase in the ratio of mtDNA:nucDNA (18 %). In contrast, the control group displayed a significant decrease in the ratio of mtDNA:nucDNA and a decrease ($p>0.05$) in CS activity (**Paper IV**, Fig. 3). This suggests that the interventions were sufficient to induce an increase in mitochondrial content.

The findings presented in **Paper IV**, indicate that training can stimulate and might improve mitochondrial function. **Preliminary data** from study 4 show a significant increase of the MEF2A protein in the resistance training group. A weak correlation was detected between ΔMEF2A and $\Delta\text{HOMA-IR}$ (as discussed in section 2.1) when pooling all intervention groups. Studies have showed that MEF2A can bind to the GLUT4 promoter and AICAR has been shown to stimulate the translocation of MEF2A to the nucleus thus affecting MEF2A regulation of GLUT4 (Holmes et al. 2005; Gong et al. 2011). No change in GLUT4 mRNA

or in the mRNA of AS160 (Akt substrate of 160 kDa, also known as TBC1D4) could be detected (**Preliminary data** from study 4). AS160 is known to be phosphorylated by AMPK as a response to exercise/muscle contraction (Cartee & Wojtaszewski 2007; Sakamoto & Holman 2008; E. A. Richter & Hargreaves 2013; Cartee 2015) and thereby initiating the translocation of GLUT4 to the cellular membrane. This ability makes it an interesting target to study in order to understand insulin-independent glucose uptake in skeletal muscle. The lack of mRNA change might be a result of timing as discussed in section 1.2, and reflects the importance of studying both the GLUT4 and the AS160 protein. In study 4, attempts to measure the protein of AS160 with ELISA was performed, but no absorbance differences compared to the blank were detected, indicating insufficient binding of the coated antibody. Also, the activation of AS160 in skeletal muscle seems to be highly dependent on phosphorylation (Cartee 2015). A regulatory stimulus, such as phosphorylation, is usually more of an acute way to regulate intracellular adaptations. However, an acute exercise bout in men has been shown to increase the levels of AS160-phosphorylation immediately after exercise, with levels still elevated 3 hrs after exercise (Howlett et al. 2008). In a 3-week one-legged training study, AS160 protein levels did not increase significantly, but the phosphorylation of AS160 was higher both before and after insulin stimulation in the leg that had trained compared with the untrained leg (Frøsig et al. 2007). This indicates that the role of AS160 in glucose regulation and in response to exercise needs to be studied with focus on phosphorylation. Also that the effect might be missed in prolonged interventions, such as that in study 4, if an intra-individual control is not included.

4.4 MITOCHONDRIAL-DERIVED PEPTIDES AFTER TRAINING IN PERSONS WITH PRE-DIABETICS

Mitochondrial retrograde signaling includes communication from the mitochondria to the nucleus as well as to other cellular elements (C. Lee et al. 2013). Transcription factors, such as PGC-1 α have been identified as factors affected by mitochondrial retrograde signaling (Chae et al. 2013). Recently, both humanin (HN) and MOTS-c have been detected and described as small peptides of mitochondrial origin that could act as retrograde signals. Interestingly, HN levels in plasma have been implied to be lower in persons with impaired fasting glucose regulation in comparison to healthy controls (Voigt & Jelinek 2016). MOTS-c has also been shown to be important for glucose regulation by activating AMPK and GLUT 4 in mice skeletal muscle (C. Lee et al. 2015). Furthermore, MOTS-c levels are reduced in

individuals suffering from obesity and in patients with insulin resistance (C. Lee et al. 2015). Both resistance training and endurance training are known to activate AMPK (Dreyer et al. 2006; Lundberg, Fernandez-Gonzalo & Tesch 2014) which in turn is activated by increased ATP turnover and modulates cellular metabolism by phosphorylating metabolic enzymes (Carling & Hardie 1989; Kahn et al. 2005). Since MOTS-c has been shown to activate AMPK it has also been speculated that it is involved in exercise adaptation (C. Lee et al. 2016). Furthermore, increased HN levels have been reported in conditions with oxidative stress such as in patients with mitochondrial encephalomyopathy suffering from lactic acidosis and stroke-like episodes (MELAS) (Kariya et al. 2005). In these types of conditions, the mitochondrial function is impaired and the altered ATP/ADP ratio is somewhat similar to the intermittent alteration in cellular homeostasis seen with acute exercise. Due to the similarities between HN and MOTS-c it can be speculated that they might act in concert.

4.4.1 Humanin expression and training

In **Paper IV**, the levels of the HN protein in skeletal muscle and in serum in men with IGR were measured. In the resistance training group, a significant increase of the HN protein in skeletal muscle was observed after the intervention compared to before, and compared to both the aerobic (Nordic Walking) and the control group (**Paper IV**, Fig. 1 A). Pooling the data from all three groups revealed a weak correlation of HN levels and HOMA-IR. Since the correlation was so weak, no firm conclusion should be drawn from the statistical interaction between HN and HOMA-IR. However, contrary to our hypothesis, the intervention seemed not to elicit any changes in the HN protein levels in serum. Although, Δ HN protein levels in serum did correlate to the improvements in the Δ 2 hrs glucose loading test in the resistance training group (**Paper IV**). This might indicate an endocrine rather than auto/paracrine effect of HN protein in serum. Interestingly, Muzumdar *et al.* have shown that injections of an HN analog in rats lowers blood glucose levels in a rat diabetic model (Muzumdar et al. 2009). The lack of change in the aerobic group (Nordic walking) could be due to the low-intensity rather the training type *per se*, something that has been discussed previously (McGarrah et al. 2016). Also, to bear in mind when conducting exercise interventions, mean weekly adherence was 1.9 and 1.8 sessions per week in the resistance and the aerobic training group respectively and not 3.0 as intended. Furthermore, it would be interesting to study the HN protein in response to training in both younger individuals and in a more demanding aerobic training set-up. Even though, **Paper IV** could not conclude any specific improvements in the

prediabetes profile in skeletal muscle, previous publications based on the Nordic Walking study, to which study 4 belongs, have shown that markers involved in a diabetic profile (Venojärvi, Wasenius, et al. 2013) as well as markers for metabolic syndrome (Venojärvi, Korkmaz, et al. 2013) were improved with the performed intervention.

4.4.2 MOTS-c expression and training

In study 4, the protein levels of MOTS-c before and after the intervention in both skeletal muscle and serum from men with IGR were measured. MOTS-c protein in skeletal muscle showed an increase (19.5 %) in the resistance training group ($p < 0.05$, $n = 15$, **Preliminary data**, see Fig. 12). Serum protein levels of MOTS-c did not change over time and there was no difference between the groups. However, correlation analysis of the change over the 12-weeks intervention (Δ values) revealed a negative correlation between Δ MOTS-c protein in serum and Δ RIP140 protein in skeletal muscle (**Preliminary data**, study 4, $r = -0.513$, $p < 0.01$). Interestingly, a positive but weak correlation between Δ MOTS-c protein in serum and Δ GLUT4 mRNA in skeletal muscle was detected (**Preliminary data**, study 4, $r = 0.359$, $p = 0.015$). This may lead to speculations that a cross-talk between of MOTS-c in serum and metabolic factors important for oxidative capacity in skeletal muscle might exist.

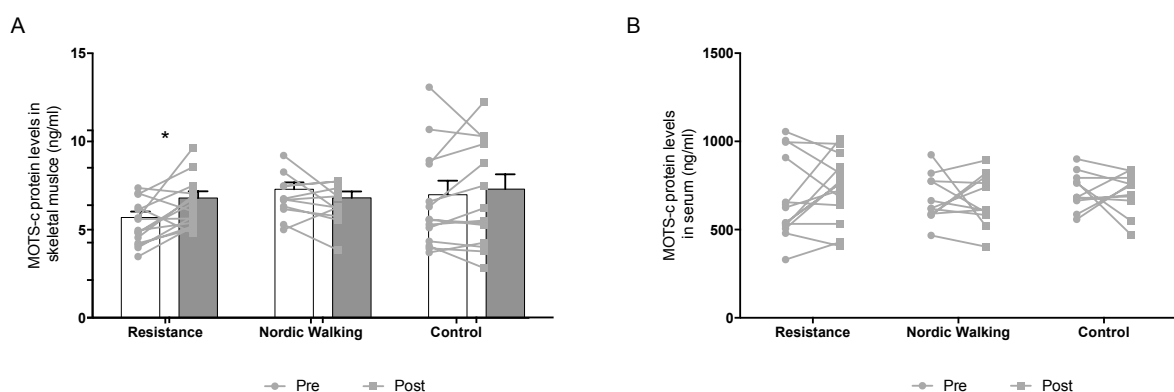


Figure 12. Preliminary data; MOTS-c protein levels in human skeletal muscle (RT $n = 15$, NW $n = 11$ and CON $n = 14$) (A), and in serum (RT $n = 14$, NW $n = 11$ and CON $n = 11$) (B) before and after 12-weeks of training, study 4. ^a $p \leq 0.05$ with factors time \times group (LMM). * $p \leq 0.05$, compared to Pre (pairwise comparison (Sidak correction) within groups). The bars represent means \pm SEM.

In RIP140-null mice, an increased fatty acid utilization and increased GLUT4 trafficking and glucose uptake in skeletal muscle have been shown (Fritah et al. 2012). RIP140 has been recognized as an important inhibitory player in the regulation of fat and glucose metabolism (Rosell et al. 2011), and T2DM patients have been observed to have higher basal levels of RIP140 (Xue et al. 2012). Also, in rodents RIP140 has been shown to act as a negative regulator of glucose uptake and to suppress expression of mitochondrial proteins (Catalán et al. 2009; Chen et al. 2012). Interestingly, mice treated with intraperitoneal injections of MOTS-c for 7 days display a significant increase in glucose clearance and improved whole-body insulin sensitivity (C. Lee et al. 2015). The same study also found that muscles from older (12 months) mice were more insulin resistant than younger (3 months) mice, but that a 7-day MOTS-c treatment restored sensitivity in the old mice to levels comparable to that in young animals (C. Lee et al. 2015). Also, overexpressing MOTS-c in L6-myoblasts revealed an accelerated glucose clearance. Further, MOTS-c treatment (72 hrs) of HEK293 cells increased phosphorylation of AMPK and ACC, and elevated CPT-1 protein levels, similar to the effects of the well-known diabetes drug metformin (C. Lee et al. 2015). However, one key difference between the actions of MOTS-c and metformin is that MOTS-c appears to directly target skeletal muscle as the key site of its activity, while metformin is thought to act primarily on the liver (Diamanti-Kandarakis et al. 2010; Viollet et al. 2012). Taken together, our findings together with earlier findings from Lee et al 2015, point toward a link between MOTS-c in serum and metabolic regulatory factors such as RIP140 in skeletal muscle of men with IGR. This makes MOTS-c a really interesting target to study further in the attempt to prevent metabolic diseases.

4.5 SUMMARY OF MAIN FINDINGS

The results of the present thesis show that both acute and prolonged exercise training can induce changes in the transcription and protein machinery, mainly coupled to improved metabolism and mitochondrial oxidation in human skeletal muscle. The studies provide a comprehensive analysis of PGC-1 α , the different PGC-1 α transcripts and related regulatory network in human skeletal muscle in response to exercise. Ischemic perturbation during exercise enhanced activation of upstream regulatory factors coupled to PGC-1 α . A one-legged exercise model provided opportunity to study intra-individual changes of factors important for mitochondrial oxidation in response to acute exercise. Time-course analysis over 24 hours gave insights into the complex fluctuations and diurnal changes that might affect some of the genes and proteins involved in the acute adaptive response to exercise. Studying mitochondrially-encoded peptides in a population with impaired glucose regulation provided insights into the tentative role of the mitochondria as “exporters” of signaling molecules contributing to exercise adaptations.

The main findings from this thesis were that;

- acute aerobic exercise and exercise with restricted blood flow strongly affect PGC-1 α mRNA and protein.
- different PGC-1 α transcripts exist in human skeletal muscle.
- different PGC-1 α transcripts display differences in base-line expression.
- acute exercise rapidly increases PGC-1 α -ex1b mRNA and protein levels.
- PGC-1 α -ex1b is the most exercise-responsive PGC-1 α transcript.
- full length BRCA1 and its splice variant BRCA1 Δ 11 and BRCA1 Δ 11b are detected in human and mouse skeletal muscle.
- exercise seems to increase the interaction between BRCA1 and phosphorylated ACC both in humans and in mice.
- there seems to be a sex difference in the BRCA1-ACCp interaction in mice.
- high fat diet in mice upregulate BRCA1 gene expression.
- reduced BRCA1 expression in human skeletal muscle cells results in increased lipid storage, decreased insulin signaling, reduced mitochondrial function and enhanced ROS production.
- the basal levels and the exercise response of MaCoA is greater in female than in male mice.

- AMPK appears to be a strong candidate for exercise-induced activation of PGC-1 α , but may not be crucial for the BRCA1 activation in skeletal muscle.
- two LIPIN-1 (LIPIN-1 α and LIPIN-1 β) and three NCoR1 (NCoR1-1, NCoR1-2, and NCoR1-3) isoforms are present in human skeletal muscle.
- the humanin protein, encoded from the open reading frame of the mitochondrially encoded 16S rRNA gene, is upregulated by resistance exercise in men with impaired glucose regulation, possibly in an intensity-dependent fashion.
- MOTS-c, encoded from the open reading frame of the mitochondrially encoded 12S rRNA gene, seems to respond in a similar fashion to exercise as humanin.

4.6 GENERAL DISCUSSION

A high oxidative capacity is important for the maintenance of health and prevention of disease. Skeletal muscle is highly plastic and a key player for whole body metabolism and its wide dynamic range of the metabolic turnover is well recognized as an important feature of a healthy individual.

This thesis examines the influence of exercise on PGC-1 α , a key regulator in the control of oxidative function. Nevertheless, the specific regulation of cell metabolism and the involvement of PGC-1 α in aging and metabolic disorders are poorly understood. Exercise has been acknowledged in the same aspects as medicine in the treatment of some diseases, and concepts such as EIM (Exercise Is Medicine) has been established.

The concept that exercise is beneficial for health is nothing new (Berryman 2010). Over 2,500 years ago, a physician called Susruta prescribed exercise as a treatment for his patients. Documents even show that T2DM was believed to be a curable disease of the urinary tract for which he prescribed a certain diet and exercise. However, according to Susruta it was important that the exercise performed was not vigorous or with high-intensity since this was regarded as a risk factor for developing multiple diseases and potentially leading to death (Tipton 2014). Later, during the Persian empire, military leaders recognized the link between soldiers with good strength and endurance with the success on the battlefield. This led to rigid training programs which included both strength, endurance and nutritional optimization (Kokkinos n.d.). And as one of the most famous exercise physicians, Hippocrates (460-370 BCE) wrote “eating alone will not keep a man well, he must also take exercise“ (Berryman 2010; Tipton 2014). In this perspective, exercise as prevention and treatment for common diseases such as T2DM is regarded as crucial, and the research field of molecular exercise physiology has evolved tremendously over the last decades. However, there are significant gaps in our understanding of the biology of exercise and its beneficial health effects and skeletal muscle adaptations.

In this thesis, human exercise studies were performed in an attempt to advance the understanding of the regulation of the adaptations that occur following both acute and prolonged exercise training. By having the opportunity to study men with impaired glucose regulation, new insights into the connections between the mitochondria and metabolic control were also achieved. However, when performing human exercise studies, it is important to be cautious and aware of the large intra- and inter-individual variability that exists in humans, which sometimes can be seen as a major limitation in studying training-induced skeletal

muscle response and adaptations. In addition, differences in genetics and epigenetics, sex hormonal profile, lifestyle choices as well as numerous physiological and psychological factors may all affect the results of the study. This is something that needs to be considered before any general conclusions can be drawn from the data. In study 1, an intra-individual control (non-exercising leg) was used, and in study 3 and 4 a control group was included. However, in study 2, inter-individual variations and sex differences, might have blunted some of the exercise effects since the number of study subjects was rather small. In study 4, the randomized groups were not fully homogenous, with the control group being more fit and also displaying lower body weight than the intervention groups. This might have influenced some of the comparisons made. And since the control group also was given information about the benefits of exercise it is quite likely that some of the included subjects were more active than before the intervention period.

Human studies provide somewhat limited mechanistic insights, why they sometimes need to be complemented with animal models and cell studies to establish more direct mechanistic relationships, as done in study 1 and 2. The results from animal studies and in vitro experiments are commonly extrapolated to humans and there is not always an agreement between the results from different species (Burkhardt & Zlotnik 2013; Vandamme 2014). This was the case in study 2, in which the animal model showed sex differences in the magnitude of interaction between BRCA1 and ACC-p, as a result of the exercise stimuli but not in the human biopsy material. The high variability seen in the human samples may be, in part, due to the large degree of genetic variation in BRCA1, which can result in altered protein function and/or expression. The phosphorylated form of ACC interacts with BRCA1 in the C-terminal region, specifically in the BRCT domains. Even though the BRCT domains in BRCA1 are well conserved across species, the BRCT domains in the human BRCA1 gene contain the highest degree of genetic variation (M. S. Lee et al. 2010).

Also, conducting human exercise studies gives access to limited amount of material and usually mRNA analysis is conducted since it is quite straight forward and does not demand high quantities of material. In this thesis, the majority of the studies quantified gene expression changes. Gene expression changes measured as mRNA expression, are not necessarily synonymous with concurrent protein or functional changes. Specific mRNA levels do not always correlate with protein levels, although a high over all correlation has been suggested (Vogel & Marcotte 2012). Usually proteins exhibit a larger dynamic range of concentrations than transcripts do, which might mainly be due to post transcriptional mechanisms as well as a cumulative “stair-case” pattern of a repeatedly activated mRNA and

protein expression (Vogel et al. 2010; Perry et al. 2010). And this dynamic range is highly individual within humans regarding its magnitude of response.

In animal models, the whole muscle is used for homogenized instead of a small biopsy as in humans. Therefore, it is also important to remember that the skeletal muscle is not a fully homogenous tissue. Possible differences in fiber type composition, and thereby differences in *e.g.* oxidative capacity, are present along the muscle length and at various depths (Vogel et al. 2010; Perry et al. 2010). Also, differences in the activation pattern and metabolism during exercise within the skeletal muscle fibers have been observed (Lexell et al. 1985). However, in all the studies conducted in this thesis, the biopsy procedure was highly standardized to minimize these types of errors. Nevertheless, when interpreting results from animal studies in the molecular exercise field, you need to remember that voluntary exercise is not always the case. Some exercise studies in animals apply an exercise stimulus that is more of a “life or death type” of situation along with substantial stress levels, which could highly influence *e.g.* the metabolic pathways aimed to be studied. Given these interspecies differences, there are clear advantages with human research. Furthermore, performing exercise studies may lead to increased understanding of skeletal muscle function and metabolism which is useful in an attempt to improve over-all health in humans.

5 CONCLUSIONS

This thesis concludes that;

- the regulation of mitochondrial adaptation following exercise is far more complex than previously thought as indicated by the presence and differential regulation of the two known PGC-1 α promoters in humans skeletal muscle.
- the PGC-1 α transcripts have different roles in the adaptational process since they respond differently to exercise and exercise mimicking stimuli.
- it is possible that the PGC-1 α -ex1b protein is involved in the initial adaptation phase since it responds rapidly to exercise.
- exercise might facilitate the mitochondrial adaptation through suppression of RIP140, a PGC-1 α corepressor.
- the improved fatty acid metabolism in skeletal muscle following exercise might be in part a consequence of increased interaction between BRCA1 and ACC-p .
- it may be suggested that humanin has a role in glucose metabolism since it increases in skeletal muscle after regular resistance training in prediabetic males.

6 FUTURE PERSPECTIVES

The human body is made to move. Watching kids play, is sometimes like observing the very cornerstones of human physiology and kinetics. Historically, these movement patterns were key for gathering food, building tools and shelter, hunting and evading predators etc. So, from an evolutionary perspective, the ability to perform exercise was essential for survival. We are made to move, so what happens when we stop? Playgrounds are one thing, but consider the type of activities that are essential for survival today. What helps us survive in a modern technological society?

In order to maintain health, it has been shown repeatedly that the human body needs a certain amount of physical activity. This need of physical activity is tightly coupled to the activation of gene programs that evolved during the time when we were required to be much more physically active than today. For many years, researchers have focused intensively on trying to understand the mechanisms behind training adaptation such as improved metabolic function and mitochondrial biogenesis. This has, in part, been done by studying how genes respond to training and lifestyle interventions. Despite substantial progress, significant knowledge gaps still remain. Thus, the mechanisms of exercise and how it affects all the cells in the body will most certainly continue to be of great scientific and public interest in the future.

The studies in this thesis have hopefully shed new light on some of the pieces of this large puzzle, but more studies are obviously needed. In the future, new genetic and molecular techniques will further develop the scientific understanding of exercise biology. In study 4, a relative novel global gene expression analysis method, RNAseq, was employed to provide further understanding of the complex gene network activated by a certain type of training and how this differs between individuals. Also, we hope to see if the degree of metabolic dysfunction (impaired glucose regulation) might affect the outcome of the training response.

Life is unfair, we respond differently to the same exercise stimulus which points towards the importance of understanding the individual response that training can cause. This will lead to the development of more personalized training strategies and can also improve the concept of exercise as medicine. Ultimately, this might lead to new strategies against several common diseases.

The magic of the mitochondria is not fully understood, and the unique structure and functional characteristics of this organelle have raised the ideas of drugs selectively targeting

the mitochondria. Also, as this thesis shows, retrograde signaling molecules like mitochondrial-derived peptides (humanin and MOTS-c) might have more biological functions than shown previously. These peptides, released from the mitochondria, have already been linked to protective effects in some diseases such as Alzheimer's disease and cancer. Exercise increases the size, number and amount of the mitochondria and increases the copy number of the mtDNA which might be of importance in producing more of these cyto- and neuro protective peptides.

Furthermore, in the light of EIM, obese individuals and patients with diabetes are characterized by impaired mitochondrial function and reduced amount of mitochondria, and the skeletal muscle PGC-1 α mRNA expression is reduced in those with diabetics. Since PGC-1 α is important in the control of energy metabolism and insulin sensitivity, it is a highly interesting target for novel drugs. Exercise or specific drugs that activate PGC-1 α with the aim to stimulate mitochondrial biogenesis should be considered for people with diabetes. PGC-1 α has also been implicated in a novel angiogenic pathway, and results from this thesis may strengthen the notion that exercise-induced activation of PGC-1 α might be important for the treatment of ischemic diseases. Collectively, further developments in the area of mitochondrial medicine and exercise medicine will be very important to progress in health and disease management.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

“If we could give every individual the right amount of nourishment and exercise, not too little and not too much, we would have found the safest way to health”

Hippocrates, ~ 450 BC

Kroppen är byggd för rörelse. Att röra på sig regelbundet har visats förbättra funktionen hos flera organ i kroppen, minska risken för sjukdomar och i vissa fall behandla sjukdom. Att vara fysiskt aktiv ökar t.ex. den fysiska prestationsförmågan, gör att vi sover bättre och till och med får fler celler i vissa områden i hjärnan som leder till att vårt minne förbättras. Dessutom är övervikt och diabetes betydligt mindre vanligt hos personer som motionerar. Det finns starka vetenskapliga bevis för att en inaktiv livsstil påverkar hälsan negativt och människor som är otillräckligt fysiskt aktiva lever mellan 6 och 9 år kortare än personer som är regelbundet aktiva.

På detta sätt kan det vara förenat med ökad risk för förtidig död att skriva en avhandling, detta då personen i fråga ofta sitter enormt många timmar av sin vakna tid under skrivprocessen.

7.1 VI OCH VÅRT DNA

Människokroppen består av ca hundra tusentals miljarder enskilda celler som samverkar för att vi ska fungera som en organism. Grunden för människan som livsform ligger i vår kod, den vi kallar arvsmassan eller DNA. Denna kod är specifik och bildas då ägget och spermien smälter samman vid befruktningen, varvid hälften av arvsmassan kommer från ägget och hälften från spermien. Från denna embryonala stamcell utvecklas en mängd olika celler och celltyper varpå sedan vävnader, organ och till sist organsystem byggs upp vilket är det som utgör dig som människa. Inuti varje cell finns en cellkärna (förutom i de röda blodkropparna) i vilken den genetiska koden/vårt DNA finns packat. I varje cellkärna ryms ca två meter DNA som kodar för ca 20 000 gener dvs. avsnitt på DNA som kodar för ett visst protein. Det kan tyckas mycket med två meter DNA, men inte om man jämför med det japanska ormbäret som innehåller nästan 100 meter DNA per cellkärna. Men lugn, packningen av vårt DNA är optimerat så det finns gott om plats. Alla celler innehåller samma genetiska material, samma DNA, men ändå kan celler ha olika funktion och utseende. Detta beror på hur koden läses av, vilket avsnitt av koden som läses dvs. vilka gener som uttrycks i just denna cell. DNA kan ses som ett referensbibliotek med massa recept eller ritningar och beroende på vilket protein vi

vill bygga så är det ett specifikt avsnitt av DNA-molekylen som läses och varje sådant specifikt avsnitt är en gen. Generna uttrycks (läses och kopieras) till en molekyll som kallas mRNA som sedan kan översättas och veckas till ett funktionellt protein i cellerna.

Vår yttre omgivning och inre miljö skickar signaler till cellerna om vilka proteiner som behövs och påverkar därmed vilken/vilka gener som skrivs av. Hur vi lever och rör på oss samt olika typer av sjukdomar kommer också att skicka sådana signaler till cellerna och påverka genuttrycket.

Förenklat kan man säga att varje gen kodar för ett enskilt protein, och det är proteinerna som står för majoriteten av kroppens olika funktioner. Proteinernas funktion är t.ex. att bygga upp våra muskler och skapa rörelse, fungera som biologiska katalysatorer och markörer, ge våra celler och kroppar struktur och hållbarhet, färga vårt blod rött och bära på syremolekyler i blodet samt möjliggöra kommunikation inuti celler och mellan vävnader.

I denna avhandling har jag studerat hur akut (ett enstaka träningspass) och regelbunden träning påverkar hur vi läser av vår kod och vilka olika mRNA molekyler som bildas samt om dessa bygger funktionella proteiner och om vissa proteiner ökar eller minskar som svar på träningen. De faktorer jag har fokuserat mest på är de som är kopplade till att öka muskelns aeroba anpassning och de som stimulerar cellen till att bygga fler mitokondrier (se förklaring nedan).

7.2 SKELETTMUSKULATUREN OCH DEN MAGISKA MITOKONDRIEN

I vår kropp finns tre olika typer av muskulatur; skelettmuskulatur, glatt muskulatur och hjärtmuskulatur. Skelettmuskulaturen är den primära vävnaden som undersökts i denna avhandling. Skelettmuskulaturen är ansvarig för alla våra viljemässigt styrda rörelser, vilket bl.a. inkluderar vårt kroppsspråk, att springa och att äta och tala. Skelettmuskulaturen har också förmågan att agera som en metabol vävnad vilket är viktigt för regleringen av glukos i blodet, blodsockret. En frisk människas kroppsvikt består till ca 40-45 % av skelettmuskulatur, vilket innebär att det är ett av våra största organ. Den är uppbyggd på liknande sätt som en rysk babushkadocka, lager på lager. Skelettmuskulaturen är uppbyggd av långa, tubformade celler som kallas myocyter eller muskelceller. Zoomar vi in i muskelcellen så ser vi att dessa ibland skiljer sig åt i funktion och utseende varpå vi kan dela

in muskelcellerna i två huvudkategorier, långsamma (typ I) och snabba (typ IIa och typ IIx). Alla muskelfibrer är uppbyggda på principiellt samma sätt och består av muskelproteiner (aktin och myosin) som har möjlighet att glida över varandra och på så sätt förkorta/kontrahera en muskel. Denna process involverar kalcium och kräver energi i form ATP (Adenosin-Tri-Fosfat). Den minsta funktionella enheten kallas sarkomer och kan ses som små, små lådor innehållande våra muskelproteiner som läggs på rad i en mycket strikt ordning. Dessa lådor bildar sedan det vi kallar myofibrill, långa trådar av kontraherande enheter. Ett stort antal myofibriller buntas sedan ihop och utgör då en muskelcell. Flera muskelceller buntas sedan ihop och flera buntar av dessa utgör sedan själva muskulaturen (se Fig. 1 i avhandlingen).

Inuti de flesta celler i vår kropp finns mycket små organeller som kallas mitokondrier. Dessa tros från början ha varit prokaryota celler (bakterier) som genom endocytos (upptag i annan cell) för ca 2 miljarder år sedan inneslöts i en eukaryotcell. Då mitokondrierna har förmåga att förbruka syre och bilda ATP utgjorde detta startskottet för utvecklingen av eukaryota organismer. Våra mitokondrier är alltså kvarlevor från en prokaryot cell och har gett oss förutsättningen till att bilda ATP genom nedbrytning av näringsämnen såsom kolhydrater och fett (genom syre krävande processer) (se Fig. 2 i avhandlingen). Mitokondrier är på så sätt cellens ”kraftverk”. Ett tydligt tecken på att mitokondrien är så speciell är att den har sitt eget DNA, så kallat mitokondriellt DNA (mtDNA). Jämfört med kärnans DNA är mtDNA mycket litet (endast uppbyggt av 16569 baspar jämfört med DNA som består av ca 3,3 miljarder baspar) och har formen av en cirkel. Skelettmuskulaturen är en av de vävnader som innehåller enormt många mitokondrier vilket skapar möjlighet för att kunna producera mycket ATP och på så sätt generera kraft och förflyttning. Andra mitokondririka vävnader är framförallt levern och hjärtat.

7.3 MUSKLER, MITOKONDRIER, GENER OCH TRÄNING

Skelettmuskulaturen anpassas efter hur vi lever, regelbunden träning gör den starkare och mer uthållig medan inaktivitet och stillasittande gör det motsatta. Mitokondrier påverkas också mycket av hur vi lever och av träning. Särskilt uthållighetsträning signalerar till kroppens vävnader såsom skelettmuskulaturen att fler mitokondrier ska byggas, så kallad mitokondriell biogenes. Träning gör att mitokondrierna blir fler och effektivare på att förbränna näringsämnen och syre, samt att bilda energi. Även om mitokondrien har sin egen genetiska kod så är den i behov av signaler/proteiner från kärnans kod för att kunna läsa av och kopiera sitt mtDNA.

Det finns otaliga studier som visar att träning, både akut och regelbunden, förändrar vilka gener i vårt DNA som skrivs av. Detta leder i slutändan till de anpassningar vår kropp åstadkommer som svar på träning vare sig det handlar om att bilda fler blodkärl, fler mitokondrier, fler muskelproteiner eller göra vårt hjärta starkare.

7.4 MUSKELBIOPSIER OCH MOLEKYLÄRA METODER

För att kunna studera vad som händer i skelettmuskulaturen på genetisk nivå så har vi i denna avhandling tagit muskelprover, så kallade muskelbiopsier, från våra frivilliga försökspersoner. Dessa biopsier kan i storlek jämföras med ett tjockt riskorn och väger ca 100 mg, vilket är en relativt försumbar bit räknat till lårmuskulaturens totala storlek. I korthet går proceduren till som följer; huden lokalbedövas, ett litet snitt görs genom huden och ner till muskelns yttre hinna, muskelfascian. Biopsinålen förs därefter ner genom snittet och in i muskeln. Biopsinålen kan liknas vid en giljotin, muskeln sugas in i en liten öppning i nålen för att sedan kapas av. Samtliga muskelprover i denna avhandling har tagits från den yttre av lårmuskulaturens fyra delar, *musculus vastus lateralis*.

Då skelettmuskulaturen är en sådan fantastisk vävnad att studera och har en enorm förmåga till anpassning så möjliggör det att vi även kunnat ta upprepade biopsier från samma person. Detta har gjort att vi kunnat studera de genetiska och mitokondriella förändringar som sker över tid med träning. För att studera detta så har molekylära analyser gjorts i vårt laboratorium, med en kombination av kemi, molekylärbiologi och modern teknik där DNA, mtDNA, mRNA och specifika protein kunnat isoleras från muskelproverna för analys. Celler som isolerats från muskelproverna har också odlats för att kunna studera hur olika tillsatta ämnen påverkar vissa gen- och proteinuttryck.

Utöver muskelprover har även blodprover tagits samt fysiologiska tester utförts på försökspersonerna såsom t.ex. max-prestationstest på cykel och test av muskelstyrka och explosivitet.

7.5 VAD VISADE EGENTLIGEN AVHANDLINGEN?

I denna avhandling har fyra olika träningsstudier genomförts (se Tabell.1 i avhandlingen). En kortfattad sammanfattning av de viktigaste resultaten följer nedan.

I studie 1 har träning med reducerat blodflöde till muskeln använts (genom att träna ena benet i en tryckkammare) för att studera hur ischemisk träning (nedsatt blodflöde som leder till bl.a mindre syretillgång) påverkar träningssvaret i skelettmuskulaturen efter 45 minuters enbens-sparks träning (ena benet tränade med ischemi och det andra utan). I studie 2 och 3 har försökspersonerna cyklat i 60 minuter, och i studie 2 användes en translationell försöksdesign vilket innebar att även möss tränades. I studie 4 genomfördes 12 veckors träning med tre grupper av män med nedsatt glukosreglering, så kallade pre-diabetiker. Dessa män delades slumpmässigt in i grupper och tränade antingen styrketräning eller stavgång alternativt ingick i en kontrollgrupp. Samtliga studier i denna avhandling studerar hur träningen påverkade olika gener och proteiner kopplade till mitokondriell biogenes med främst fokus på ett protein som heter Peroxisome proliferator-activated receptor (PPAR)- γ coactivator (PGC)-1 α som vi kallar PGC-1 α .

Resultat från studie 1 visar att ischemisk träning påverkar både gen- och proteinuttrycket mer än traditionell konditionsträning. Vi kunde också för första gången hos människa visa att det finns isoformer (varianter) av PGC-1 α som skrevs av från andra promotorer (avsnitt på DNA som reglerar gener uttryck), dessa kallas PGC-1 α -ex1a och PGC-1 α -ex1b. Vi kunde med cellförsök också se att en av dessa PGC-1 α varianter, nämligen PGC-1 α -ex1b är den som svarar mest och snabbast på träning. Analyser gjordes både i homogenat och från odlade celler från muskelproverna. I studie 2 undersökte vi hur akut cykelträning (försökspersoner) eller löpning (möss) påverkade ett protein som heter breast cancer 1 early onset (BRCA1), sedan tidigare mest känd inom bröstcancerområdet. Vi ville se hur detta östrogenkänsliga protein är kopplat till fettmetabolismen och mitokondriell funktion. I denna studie kunde vi i möss se att BRCA1 uttrycks olika i olika typer av muskler samt att uttrycket skiljer sig mellan könen. I människa kunde vi se att träning påverkar och ökar interaktionen av BRCA1 med fosforylerat acetyl coenzym A carboxylas (ACC-p) vilket ökade fettmetabolismen och är kopplat till förbättrad mitokondriell funktion samt ökad insulinkänslighet.

I studie 3 ville vi studera hur PGC-1 α och dess olika isoformer (fem olika varianter) påverkades av ett enstaka träningspass samt ett nätverk av faktorer kopplade till PGC-1 α och mitokondriell biogenes. Vi ville också studera hur gen- och proteinuttryck förändrades som svar på träning över 24 timmar. Detta gjorde vi genom att ta upprepade biopsier (5 st) i en grupp som randomiserats till träning och en grupp som inte tränade (kontroller). I denna studie kunde vi återigen se att PGC-1 α -ex1b isoformen är den mest träningskänsliga av PGC-1 α varianterna och att den verkar vara inblandad i det initiala adaptationssvaret som sker efter ett enstaka träningspass. Vi kunde också se att vissa genuttryck som tidigare vetenskapliga

studier föreslagit som ”tränings-gener” egentligen var aktiverade som en följd av själva biopsitagningen.

I studie 4 ville vi studera hur olika typer av träning (styrketräning eller stavgång) påverkar muskulaturens metabolism och reglering av glukos hos män i åldern 40-65 år. I denna studie visar vi också att mtDNA verkar kunna producera två egna proteiner som kodats från det lilla mtDNA:t. Dessa proteiner har inte helt kartlagda funktioner men troligtvis är de kopplade till insulinkänslighet, åldrande och innehar cellskyddande egenskaper och har setts påverka miljön utanför mitokondrien. Dessa proteiner heter humanin och MOTS-c och verkar kunna skrivas av och öka i proteinmängd med främst styrketräning. Dessa mitokondriellt kodade proteiner kan vara viktiga för den förbättring som ses av glukosregleringen med träning, men har också visats skydda nervvävnad och motverka delningen av cancerceller, och kan därför vara viktiga i syfte att minska risken för att drabbas av typ 2 diabetes eller andra sjukdomar såsom Alzheimers och cancer.

8 ACKNOWLEDGEMENTS

First, I would like to thank all the fantastic **volunteers** who participated in all my studies. I am so grateful for your generosity and your braveness, by letting us take your time and muscle for scientific purposes. Thanks also to all the **animals** who sacrificed their lives in our attempt to understand the metabolic role of BRCA1! Without you all, none of this would have been possible.

I wish to express my sincere gratitude to everyone that has contributed to this thesis.

Especially, I wish to thank; *My main and magnificent supervisor*, **Carl Johan Sundberg** for providing an inspiring and creative work environment. Your enthusiasm and generosity is unbeatable. I will always remember the first time I met you (my first day at KI as a student) when you manage to penetrate the floor and stood with your leg stuck at the height of your knee... You are not only the best supervisor one could wish for but also the greatest boss, mentor, psychologist, doctor and enthusiastic training adept one could ever have. You are a good listener and you see people for what they truly are but also what they can be. I will always be grateful for the faith you have put in me, and the self-confidence that you have given me over these years. You have taught me a lot. Thank you so much for the great experience of doing a PhD in your group.

My fantastic co-supervisors: **Jessica Norrbom**. My friend and co-supervisor a.k.a. Super Mom. Your dedication and commitment to science, teaching and exercise is something I really admire and find very inspiring. Without you this would not have been accomplished, I truly mean it. Thank you for all the great input and for always being there, you are a great listener and a true mentor. You are one of the most considerate and unselfish persons I know. Through ups and downs you have been a great support and I will always remember that. I am so impressed by all of your divergent skills and also for running marathons, running a family, running a company and running our research group with perfection. **Ulf Risérus**. I will always appreciate your many great ideas, your input and feedback. Your expertise is impressive and of great importance for this thesis. You have a way of making people feel comfortable, which I truly admire, and I hope I can do the same for any future mentees of my own.

All my co-authors; Thank you, I have really enjoyed working together.

Especially, **Kathryn “Katie” Jacksson**, for being such a good friend for a lonely swede in the U.S. For your impressive engagement to science and your biochemical skills. Thanks also for improving my everyday morning coffee. **Espen Spangenburg**, for being a great mentor during my time at UMD. Your molecular knowledge and your great humor (even though you have some Norwegian DNA in you...). **Thomas Gustafsson**, I will never forget the first time I met you, during my time as a master student you came in 15 min late with blood on your scrubs and held one of the best lectures I had during my studies at KI. You always keep impressing me with your knowledge and ideas. **Helené Rundqvist**, your knowledge and your dedication to research is impressive and I admire it a lot. **Helené Fischer**, your great willingness to help. For not laughing too hard at my stupid questions and for letting me know that it is ok to fail sometimes...and for putting your glasses in the freezer. **Ferdinand von Walden**, from “elektivansvarig” to colleague, friend and now also collaboration partner. Father of three, teacher, scientist and medical doctor doing your internship, you still find motivation to push exercise research forward, that I will always admire. Thanks for all great discussions and laughter we shared. **Björn Alkner**, thank you for good and fun collaborations and your generosity. You are the one that will put Eksjö on the Swedish map! **Mika Venojärvi**, thanks for a good collaboration in the Nordic Walking study.

Great colleagues and friends;

Especially, **Mia Ydfors**, thank you for being such a good friend. For making work fun, for all our joint training sessions and discussion about life. All the fun we have had at work but particularly outside of academia. I really appreciate everything we have done together and all the help you have given me during our years together at KI. We have very different personalities, you and I, and I admire your calm, clearness and patience. Working with you has made me a better scientist. Thank you so much for putting up with my stupid questions and computerdyslexia. Thank you also for being a “Skatteverket support”, without you my company would have been accused of tax-fraud. You are one of the smartest persons I know and I hope that our paths will cross again in the future and that we will always be friends. **Maléne Lindholm**, it has been a true pleasure working with you, but the shoes you left were too big for me to fill... Your scientific work will always be something I respect and you have over and over again truly impressed me with your work and skills. Also, thank you for all the fun parties and for teaching me about life, politics, molecular methods, wine and how to do a nice lap dance.

Susanna Appel, Thank you so much for all the fun that we have had. For great scientific discussions, for being there in good and bad, for great laughs and lastly for teaching me about makeup and clothing ☺. Your engagement in teaching has always inspired me and you have pushed me to be a better teacher. Your method skills are always something I will be slightly jealous about, but please do not tell anyone...**Charlotte Schönbeck**, fast as a shark both on the track and in making stuff happen, a true “doer”. Thank you for keeping the whole lab organized. You are a great listener and friend and I really appreciate everything we have done together, at work and outside. **Mark Chapman**, how old are you, truly? Youngest in the lab but with impressive CV and knowledge. Your kindness, scientific engagement and smartness is admirable as well as your bad humor. I learned a lot from you. Thank you also for teaching me about the importance of having clean shoes and why you should dress in non-training cloth at work. Hope our scientific path will cross in the future. **Stefan Reitzner**, thank you for letting me provoke and tease you, I have always wanted a baby brother... It is going to be a true pleasure following your PhD and your scientific career. Eat, sleep, train, repeat. Your training motivation and dedication will always be something that I am jealous about. **Sara Mijwel**, for always being so positive. Your calm and patience has numerous times blessed the environment at the office. I am impressed about your hard training and you know that I would do anything to have your hair ☺ . I know that your PhD will be a true success and I hope we can work together in the future. **Helena Wallin**, a.k.a. 36an. Thanks for the fun times we had, mostly outside the academia, and for sometimes letting me at least win one or two balls but never a full game in squash. Thanks also for our joined teaching experience in Vietnam trying to spread the fact that “Exercise is Medicine”. **Sandra Omidvar**, you are crazy, smart and almost annoyingly strong. Always positive and just awesome, so young (10 y younger than me... ???) yet so impressive. Hope that you will stay within the exercise field in the future and that our path will cross again. **Joel Svensson**, thanks for a lot of fun and intellectual and very non-intellectual discussions. **Chang Liu**, thanks for always being so helpful in the lab.

Past and present colleagues at FyFa;

Ellinor Kenne, for being an excellent mentor and a good friend. For sharing all your experience and thoughts and for providing me with a lot of teaching experience. You are a big source of inspiration. **Lars Karlsson**, a.k.a Larkorv, thank you for all the fun we have had. In another universe and in another time, we will meet again, if not already? Thanks also to the **Lindbom** group; **Joel Rasmusson**, **Anne van der Does** and **Erik Lundeberg**. All the

scientists at FyFa who understand that the muscle tissue is the shit; **Håkan Westerblad, Johanna Lanner, Niklas Ivarsson**. Also, thanks to **Rodrigo Fernandez Gonzalo**, for all the fun we have had during all our joint conferences, you stunned me with your brilliance and I know that you will return to Sweden and kick ass in the field of exercise physiology. Thanks to senior scientists, the “oldies”; **Lennart Lindbom, Bo Rydqvist, Mats Rundgren, Lars Gustafsson, Dag Linnarsson, Stefan Eriksson** and **Jan Lännergren** for interesting lunch conversations and sharing your life experiences. Thanks to the most important people at the department who makes all the teaching possible, **Sofia Pettersson, Eva Lindgren, Maximilian Carlsson Norlin, Karariina Välimäki** and **Peter Wolf**. Without you I would have been lost. **Sofia Pettersson**, also a big thank for being a true friend and training partner. The financial administration, especially **Camilla Fors-Holmberg**. **Marie-Louise Sundin** for all your help with courses and other administrative stuff. Thanks also to **Irène Lund, Robert Frithiof, Lena Norrbrand, Micke Elm, Anna Feldrich, Elisabet Sterner-Victorin** and **Anders Arner** for important discussions regarding zebra fishes, teeth and oral microbiomes, night vision goggles, pig kidneys, how to anesthetize a sheep, pain, burglary and kite surfing.

And the biggest of thanks to all of you that I might have forgot to thank.

Past and present members of the “muscle lab” at Labmed Huddinge; **Eva Jansson**, for sharing your experience, expertise and knowledge within the exercise physiology field. **Anna Strömberg**, for fun and intellectual and un-intellectual discussions. I admire your hard work and motivation through all your years of studying for different degrees. Thank you also for correcting all my miss-spellings. **Håkan Rundqvist**, for great fun both at work and outside. Thanks also for all our discussions regarding training and for pushing me to start my own company. **Tommy Lundberg**, for always making me laugh and for all the scientific discussions we have had and the more or less insane ones. Thanks also for pointing out my love towards Mia in such a clear way ☺. Thanks also to **Anna Wiik, Erik Rullman, Mats Lilja, Seher Alam, Ted Österlund, Karl Olsson, Barbara Norman, Mona Esbjörnsson, Maria Westerståhl, Elisabeth Berg, Majken Lidén** and **Andreas Montelius**.

All my wonderful friends, especially you **Lina Halldin**. Without you I would have been lost so many times. You are my sister in arms and my forever deepest friend. You have been with me through good and bad times and I know that I always can trust you for speaking your honest mind. Thanks for your support, guidance and positivism and for always pushing me to be a better version of myself. You are of great value to me and I love you dearly.

“**Tisdagsklöbb´n**”, thanks for all your support and for all the crazy times we have had and for all your children that gives me so much joy.

“**Juntan**”, from DUKA and beyond, you will always be my true friends and F2F-discussion-forum, and thanks for always speaking your minds and letting me pick your brains.

To all the members of the **Sällstedt**, **Gidlund**, **Landervik**, **Swenson** and **Nylén** families for great support over the years, especially you **Dad** and **Mom**.

Dad, I wish you got the chance to see this book, I know that you would not have read it but that's ok...the only thing I ever saw you read was the newspaper, always in the opposite order from the end to the beginning. Thank you for always believing in me, bragging about me and making me blush even though I did not deserve it. For your tremendous kindness and all the laughter we shared. All the times you drove me to practice or to a game. I really miss you, your hugs and all the phone calls with fun discussions we have had over the years. Sometimes I truly think I still can hear you whistling. You were a true fighter and I will always admire that. Nothing was ever too hard for you. Wherever you are, I want you to know that you are always with me in my heart.

Mom, I have no words that can express my gratitude to you. All your support during the years growing up and during all of my years studying. Your warmth, kindness and love is beyond what I deserve. Your patience, determination and your curiosity to learn new things (if not connected to IT) is admirable and inspiring. Your knowledge about history is undisputable but I am so glad that you have found a new interest in genetics and the magic code that is called DNA. You are a fantastic grandmother and you have a heart the size of an ocean.

My dear **brother Marcus**, 12-years older than me, you have managed the role not only as a brother but also as a mentor and sometimes my extra dad. Thank you for letting me be totally honest when we discuss life even though we usually not agree on everything. I know that I can always count on your support. My dear **sister Hanna**, thank you for all the love and warmth, your positive thinking and for believing in me even though you have no clue what I am doing ☺. I love your attitude towards life, that everything is possible for everyone! Your creativity is an important contribution to the Sällstedts family.

My extended family, thank you for all your support and patience, I know that I have missed some important family events due to my work engagement. Thank you for all the laughter we

shared, vacations and more or less crazy dinners. Thanks for putting my life in perspective. You will always have a place in my heart. Also, an extra thanks to my mentor **Eric Nylén**, for your contributions to my scientific development and for welcoming me with open arms. You may be one of the few who actually read this thesis. For making me understand that worlds like “utomjording” is quite fun when you come to think of it and for sticking to 1970s Swedish language. Thanks also for providing me with interesting books and for opening my eyes to important stuff outside academia. You and your family have made an important imprint on my life.

Tobias, my love, my husband and dearest friend.

I was 18 years old when I met you. The most important meeting in my life, and it was not even scheduled!

Thank you for always believing in me, your support, love and kindness. All the laughter we share and the life we have built together.

I so enjoy experiencing the world together with you and hope we will continue to build long lasting memories.

Lastly, thank you for making me a better person. For lifting me up when I was down and for carrying me when times have been tough.

This thesis is also yours.

Tuva-Li and **hopefully all you future kids**, du/ni är det enda som verkligen betyder något här i livet. Visste inte hur mycket jag kunde älska någon förrän du/ni kom. Tack för att du/ni gör mig mer klarsynt och mindre jobb-sugen.

Lastly never forget that;

Det är bättre att ta ut en seger i förskott än att aldrig få fira (citat Lars-Gunnar Bengtsson), och kom ihåg att alla har det svårt ibland, så möt människor med en positiv attityd och ett leende.

Sist men inte minst, att skriva en avhandling är förenat med ökad risk för förtidig död så stå upp när du skriver den!

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